Direct Growth Suppression of Myeloid Bone Marrow Progenitor Cells But Not Cord Blood Progenitors by Human Cytomegalovirus In Vitro

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Recently, considerable interest has arisen as to use cord blood (CB) as a source of hematopoietic stem cells for allotransplantation when bone marrow (BM) from a familial HLA-matched donor is not available. Because human cytomegalovirus (HCMV) has been shown to inhibit the proliferation of BM progenitors in vitro, it was important to examine whether similar effect could be observed in HCMV-infected CB cells. Therefore, the effect of HCMV challenge on the proliferation of myeloid progenitors from BM and CB was compared using both mononuclear cells (MNC) and purified CD34+ cells. A clinical isolate of HCMV inhibited the colony formation of myeloid BM progenitors responsive to granulocyte-macrophage colony-stimulating factor (CSF), granulocyte-CSF, macrophage-CSF, interleukin-3 (IL-3) and the combination of IL-3 and stem cell factor (SCF). In contrast, colony growth of CB progenitors was not affected. In addition, HCMV inhibited directly the growth of purified BM CD34+ cells responsive to IL-3 and SCF in single cell assay by 40%, whereas the growth of CD34+ progenitors obtained from CB was not suppressed. The HCMV lower matrix structural protein pp65 and HCMV DNA were detected in both CB and BM CD34+ cells after in vitro challenge. However, neither immediate early (IE)-mRNA nor IE proteins were observed in infected cells. Cell cycle examination of BM and CB CD34+ cells revealed that 25.7% of BM progenitors were in S + G2/M phase whereas only 10.7% of the CB progenitors. Thus, a clinical isolate of HCMV directly inhibited the proliferation of myeloid BM progenitors in vitro whereas CB progenitors were not affected. This difference in the susceptibility of CB and BM cells to HCMV may partly be caused by the slow cycling rate of naive CB progenitors compared to BM progenitors at the time of infection.

HUMAN cytomegalovirus (HCMV) produces usually mild or asymptomatic infections in individuals with normal immune responses, whereas it may cause serious disease in immunosuppressed patients. Clinical manifestations include suppression of myelopoiesis, a mononucleosis-like syndrome, hepatosplenomegaly, lymphadenopathy, thrombocytopenia, and hemolytic anemia. In patients undergoing bone marrow transplantation (BMT) HCMV remains the most common infectious cause of morbidity and mortality. In addition, HCMV in BMT has been associated with delayed platelet engraftment and has been implicated as a cause of graft failure, but the pathogenesis of this suppression is not fully understood. Especially, several in vitro studies examining the effects of HCMV on bone marrow (BM) progenitor cells have shown suppression of colony formation by various putative mechanisms. For example, Sing and Ruscetti found that infection of BM cells with HCMV resulted in a decrease in the proliferation of hematopoietic progenitors. This depression correlated with an abortive infection of a small proportion of BM cells, but no evidence of productive virus infection was seen in colony forming cells. In accordance with this, Maciejewski et al have recently shown that CD34+ cells expressed HCMV immediate early (IE) gene after HCMV challenge in vitro and they observed a moderate inhibition of colony formation. In contrast, others have reported that HCMV-infected BM stromal cells or T lymphocytes do play a major role in the HCMV-induced inhibition of progenitor growth in vitro. Thus, the pathogenesis of HCMV-mediated myelosuppression is complex and could involve either a direct effect of HCMV on marrow progenitors, an alteration in the ability of BM accessory cells including T-cells to support progenitor growth, or a combination of both a direct and an indirect effect of HCMV.

Transplantation of HLA-matched BM is currently the treatment of choice for selected patients with aplastic anemia, leukemia, and severe inherited disorders. However, the availability of HLA-identical sibling donors limits this option to roughly a quarter of otherwise suitable patients. In 1989, Broxmeyer et al suggested that cord blood (CB) contained sufficient hematopoietic stem cells to serve as transplant, and now approximately 40 CB transplantations have been performed in children worldwide. Because the number of stem/progenitor cells in CB is comparable with that associated with successfully transplanted adult BM, CB transplantation may be used also in adults.

Because HCMV infection is a major problem in BMT, we decided to examine whether HCMV had the potential to inhibit CB hematopoietic progenitor growth in the same way as has been reported for BM progenitors. In the present study colony formation of hematopoietic progenitors from BM and CB was investigated using mononuclear cells (MNC) infected with a clinical isolate of HCMV in vitro. Furthermore, the direct effect of HCMV on purified CD34+ cells was examined in single cell assay.

MATERIALS AND METHODS

Cells. BM was obtained from healthy adult donors. Umbilical venous heparinized CB was collected from placentas of term newborn infants immediately after delivery and normal labor. Informed consent was obtained using protocols approved by The Institutional

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Submitted November 2, 1994; accepted May 21, 1996.

Supported by the Norwegian Cancer Society and the Norwegian Research Council for Science and Humanities.

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Review Board. Both BM and CB were centrifuged on Lymphoprep (density, 1.077; Nycomed Pharma AS, Oslo, Norway) and the light-density MNC fraction was obtained. CD34⁺ hematopoietic progenitor cells were isolated from BM and CB MNC using immunomagnetic Dynabeads M-450 (Dynal, Oslo, Norway) covered with murine monoclonal antibodies (MoAbs) against CD34. The Dynabeads were detached from the selected cells by incubation with antiserum against murine Fab fragments (DECTAHHaBEAD; Dynal) and free cells were washed and used in the described hematopoietic assays.

Viruses. For the preparation of virus stocks and titration of virus, low passage human embryonic fibroblast (HE) cells were used. Low passage (<5) wild-type strain of HCMV was propagated at low virus to cell ratios to avoid generation of defective particles. The cell culture was obtained when extensive cytopathogenic effect was evident, and centrifuged at 5,000 rpm for 1 hour. The cells were sonicated, and the cell debris was removed by centrifugation, first 2,500 rpm for 10 minutes before the supernatant was centrifuged at 11,000 rpm for 5 minutes. Viral infectivity was examined by a plaque assay. All preparations of virus stocks were produced in the same manner. Cells and virus stocks were routinely tested for mycoplasma and endotoxin contamination and found to be negative. Mycoplasma testing was performed by DNA staining with bisbenzimide (Hoechst 33258; Behring Werke AG, Marburg, Germany). Endotoxin testing was performed by means of the QCL-1000 chromogenic endpoint LAL-kit from Bio Whittaker (Walkersville, MD).

Inoculation of hematopoietic cells with HCMV. BM and CB MNC and CD34⁺ cells were infected with mock or HCMV at 1.0 pfu per cell for 16 hours at 37°C by continuous movement. The infectious dose was chosen on the basis of previous experiments showing a maximal inhibition of the growth of hematopoietic cell lines without influencing the viability during the observation period. The cells were washed twice before further use. No characteristic HCMV cytopathology or other morphological changes were observed during the infection period. The percentage of viable virus-challenged cells did not differ significantly from that of mock-injected cells as tested by trypan-blue exclusion (data not shown).

Cytokines. Purified recombinant human interleukin-3 (IL-3) and granulocyte-macrophage-colony stimulating factor (GM-CSF) were obtained from Sandoz Pharma Ltd (Basel, Switzerland). Purified recombinant human G-CSF and recombinant stem cell factor (SCF) (c-kit ligand) were kindly provided by Dr I. McNiece (Amgen Corp, Thousand Oaks, CA) and M-CSF was a gift from Cetus Corp (Emeryville, CA).

 Colony growth. Mock- and HCMV-infected MNC from BM and CB cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal calf serum (GIBCO, Life Technologies Ltd, Paisley, Scotland), 1% dextrose, bovine serum albumin (Sigma Chemical Co, St Louis, MO), 1% L-glutamine, and antibiotics (complete IMDM). 5 x 10⁴ or 1 x 10⁵ cells were plated in 35 mm Lux petri dishes (Nunc, Inc, Naperville, IL) in 1 mL of complete IMDM, with 0.3% Seaplaque agarose (FMC Bioproducts, Rockland, ME) and predetermined optimal concentrations of G-CSF (30 ng/mL), M-CSF (1,000 μM/mL), IL-3 (30 ng/mL), SCF (100 ng/mL), or a combination of IL-3 and SCF. Dishes were incubated in a fully humidified atmosphere at 37°C in 5% CO₂ for 14 days before scoring for growth of colony-forming unit-culture (CFU-c) (>50 cells). Single cell proliferation assays were performed in Terasaki plates (Nunc, Roskilde, Denmark) at a concentration of 1 cell per well in 20 μL of complete IMDM containing IL-3 (30 ng/mL) and SCF (100 ng/mL). The frequency of responding progenitors (>10 cells/well) was determined after 10 days of incubation, with at least 300 wells scored per group.

Antibodies. A mixture of mouse MoAbs C10 and C11 (Biotest, Dreieich, Germany) against the HCMV lower matrix phosphoprotein (pp65), an MoAb against a common epitope of HCMV IE1 and IE2 (E13; Biodabs, London, UK), and control mouse ascites, clone NS-1 (Sigma), were used as primary antibodies. Alkaline phosphatase antialkaline phosphatase (APAAP) method. Controls without primary antibodies were always included.

 mRNA and DNA isolation. Poly-A+ RNA was isolated from mock- and HCMV-infected CD34⁺ BM and CB cells after 3 days incubation in optimal growth conditions (IL-3 + SCF), and from colonies formed in soft agar after 14 days. HCMV infection of A549 cells, which are semipermissive for HCMV, i.e., allow IE gene expression, and HCMV infection of permissive HE cells were used as positive sample controls. HCMV DNA was used as positive PCR control and distilled water was used as negative PCR control. The cells were procured in a guanidine thiocyanate solution (4 mol/L guanidinium thiocyanate, 0.1 mol/L Tri-HCl, pH 8.0, 1% dithiothreitol, 0.5% lauryl sarcosinate) and mRNA was isolated using Dynabeads coated with oligo (dT)₃, following the manufacturer's instructions (Dynal). Total cellular DNA from cells and colonies in soft agar was extracted, after cell lysis with the guanidine thiocyanate solution by phenol:chloroform:isoamyl alcohol (25:24:1) followed with isopropanol precipitation of the nucleic acids.

Reverse transcription (RT) and polymerase chain reaction (PCR). cDNA from 10⁷ cells was synthesized in 20 μL reaction mix (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 4 mmol/L MgCl₂, 0.25 mmol/L of each dATP, dGTP, dCTP, and TTP (Pharmacia Biotech, Piscataway, NJ), 2.5 μmol/L random hexamer primers (Medprobe, Oslo, Norway), 10 U reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase; Pharmacia) and 10 units of RNAse inhibitor (RNAGuard, Pharmacia) by incubation for 15 minutes at 42°C and for 5 minutes at 95°C and then cooled to 4°C. A nested PCR-strategy was performed using primers, which span splice sites in the major immediate early (ME) gene of HCMV between exon 2 and 4. The outer sense primer was MIE exon 2, 5'-CAGACAGATGGAGTCCGTGC-3', and antisense primer MIE exon 4, 5'-CCGGTCTGGGATATTTTC-3', and the inner sense primer was MIE exon 2, 5'-CAAGAGAAGATTGAGACCTG-3', and antisense primer MIE exon 4, 5'-GTGTCCTCTGATATTTCC-3'. DNA was amplified in a total volume of 100 μL reaction mix (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.06 mmol/L of each dATP, dGTP, dCTP, and TTP (Pharmacia), 25 pmol of each of the two outer primers and 1 μL Taq polymerase (Perkin-Elmer Cetus). The nested PCR used 5 μL of the primary PCR product as a template in a 100 μL reaction mix identical to that for the primary PCR. The samples were kept at 94°C for 5 minutes before 25 cycles of denaturation at 95°C for 1 minute, primer annealing at 55°C for 1 minute, and primer extension at 72°C for 1.4 minute and a final extension for 5 minutes at the end. The nested amplification cDNA product was 275 bp long, and the nested DNA product was 579 bp long.

DNA staining. Purified CD34⁺ BM and CB cells were resuspended in ice cold 70% ethanol, incubated on ice for 30 minutes, washed twice in ice cold PBS containing 0.5% Tween 20 and stained with 0.1 mg propidium iodide (PI) per mL PBS for 30 minutes. The PI fluorescence was measured using flow cytometry (FACScan; Becton Dickinson, Mountain View, CA) and fluorescence was collected on a linear scale and cell cycle phase was assessed by the Lysis II software (Becton Dickinson).
RESULTS

The effect of HCMV on the growth of hematopoietic progenitor cells. To compare the effect of HCMV on hematopoietic BM and CB progenitor cell growth in vitro, soft agar colony assay was performed. Mock- and HCMV-infected BM and CB MNC were incubated with predetermined optimal concentrations of G-CSF (30 ng/mL), M-CSF (1,000 U/mL), GM-CSF (30 ng/mL), IL-3 (30 ng/mL), or a combination of IL-3 (30 ng/mL) and SCF (100 ng/mL) in soft agar for 14 days before scoring for colony growth. Consistent with other reports, we found that HCMV inhibited BM progenitor growth in colony assay (Fig 1). The clinical HCMV isolate inhibited BM colony formation ranging from 40% to 75% depending on the hematopoietic growth factors (HGFs) used (Fig 1). Especially, the colony formation of BM MNC induced with more lineage specific myeloid HGFs as G-CSF and M-CSF was inhibited less (not statistically significant) than the colony growth in response to more multipotential HGFs as GM-CSF, IL-3, and a combination of IL-3 plus SCF (Fig 1). In contrast, IL-3-induced CB progenitor growth was inhibited by 7%, whereas the colony formation of CB cells in response to the combination of IL-3 and SCF was not significantly affected by HCMV infection (Fig 1). Similar results were obtained using the laboratory strain AD169 (data not shown). Compared with BM progenitors, G-CSF, GM-CSF, and M-CSF showed limited capacity to induce colony growth of hematopoietic CB progenitors in vitro and the effect of HCMV was therefore not examined. Thus, HCMV highly inhibited BM progenitor cell growth, whereas CB progenitor cells were not significantly affected.

The direct effect of HCMV on the growth of CD34+ progenitor cells. In colony assay HCMV could inhibit the colony formation by directly suppressing the proliferation and differentiation of colony forming cells or indirectly through perturbation of accessory cells. To assess the ability of HCMV to directly inhibit progenitor cell growth, the myelosuppressive effect of HCMV in vitro was examined using purified CD34+ progenitors in single cell assays. The number of IL-3 plus SCF responding mock- and HCMV-infected BM and CB progenitors was determined 10 days postinfection. The results showed that the clinical isolate of HCMV inhibited the growth of purified BM CD34+ cells responsive to IL-3 in combination with SCF by 40% compared with mock-infected cells (Fig 2). In contrast to BM CD34+ cells, and consistent with the results observed using CB MNC in soft agar assay, the growth of single CB CD34+ cells was not significantly affected after HCMV infection (Fig 2).

HCMV infection in BM and CB cells. The observed difference in the ability of HCMV to inhibit the growth of hematopoietic progenitors from BM and CB could be due to a difference in the uptake of HCMV by these cells. Therefore, nuclear pp65 expression was examined in immunohistochemistry assay to determine whether the clinical isolate of HCMV was able to enter purified CD34+ CB and BM
Fig 3. Expression of HCMV lower matrix structural protein pp65 in CD34+ CB cells. Mock- and HCMV-infected CB cells were fixed for 10 minutes in acetone 3 hours after virus inoculation. The cells were incubated with the primary antibody anti pp65 for 30 minutes at room temperature and stained by the APAAP method as described in Materials and Methods.

Three hours after HCMV inoculation the HCMV specific 65 kD lower matrix early structural protein (pp65) was detected in the nucleus of about 1% of both CD34+ BM (data not shown) and CB cells (Fig 3). Thus, despite the ability of HCMV to only inhibit progenitor growth of BM cells, HCMV was taken up by CD34+ cells purified both from BM and CB.

RT of mRNA followed by PCR of cDNA was used to examine whether the uptake of HCMV by progenitor cells was followed by transcription of viral genes. Mock- and HCMV-infected CD34+ BM and CB cells were incubated in medium containing optimal doses of IL-3 and SCF for 3 days after virus challenge before examining HCMV IE expression. We were not able to show a PCR product corresponding to the cDNA of the spliced form of the MIE mRNA of 275 bp, neither in HCMV-infected BM cells nor in CB cells. In contrast, the 275 bp product was observed in both the HCMV-infected permissive control cell line HE and semi-permissive control cell line A549 (Fig 4). PCR analysis confirmed the presence of HCMV DNA in both infected CB and BM CD34+ cells but not mock-infected cells (Fig 4). The band at 579 bp represented the amplified product from HCMV DNA (Fig 4). In addition, using primary antibodies against HCMV IE1 and IE2 antigens in an immunohistochemistry assay we were unable to show HCMV IE-proteins neither in BM nor in CB cells cultured for 3 days (data not shown).

Total cellular DNA and mRNA were also extracted from colonies of mock- and HCMV-infected BM and CB progenitors responsive to IL-3 plus SCF in soft agar to determine whether the cells harbored viral DNA and expressed HCMV IE mRNA 14 days after infection. Consistent with the observations of the short time cultured cells, HCMV DNA (the 579 bp band) but not MIE mRNA (the 275 bp band) was detected in the examined colonies (Fig 4). Taken together, HCMV DNA but not MIE mRNA was observed both in infected CB and BM cells.

Cell cycling status of hematopoietic progenitors. HCMV replication and gene expression are dependent on the cell cycling status. Therefore, because no difference in HCMV infection between BM and CB progenitor cells was observed, a difference in cell cycling status at the time of infection could explain the difference in susceptibility for HCMV between BM and CB progenitor cells. Purified CD34+ BM and CB cells were stained with PI and examined for cell cycle activity using flow cytometry. The mean values for BM and CB hematopoietic progenitors in S + G2/M-phase were 25.7% ± 2.9 (n = 3) and 10.7% ± 2.2 (n = 3), respectively. Thus, consistent with the observed difference of HCMV to suppress CB and BM myelopoiesis in vitro, BM progenitors showed 2.5-fold higher cell cycle activity than CB progenitors.

The effect of HCMV on the growth of cell cycle active CD34+ CB progenitor cells. To investigate whether the quiescence of naive CB progenitor cells at the time of infection could be one explanation for the resistance to HCMV in vitro, we initiated the HCMV infection after the induction of the cell cycling of CB progenitors. The cell cycle status of purified CD34+ cells was changed by incubating the cells with a combination of the hematopoietic growth factors, IL-3, SCF, and G-CSF for 24 hours. Examining the cell cycle activity revealed that the mean value for CD34+ CB cells in S + G2/M-phase was increased from 11.5% ± 0.3 at the time of harvest to 29.9% ± 3.3 (n = 2) after 24 hours with cytokine stimulation. Mock- and HCMV-infected cell cycle induced CB progenitors were seeded in soft agar colony assay. The colony formation of the cell cycle active CD34+ CB cells in response to SCF, IL-3, and G-CSF was inhibited by the clinical isolate of HCMV by 64% ± 22 (n = 4). However, comparable to the effect of HCMV on prolifer-
tion of naive CD34⁺ CB cells in single cell assay (Fig 2), the colony growth of CD34⁺ CB cells infected with HCMV before induction of cell cycle was not significantly inhibited (data not shown). Thus, the observed difference in susceptibility to HCMV between naive CB and BM progenitors could be caused by the slow cycling rate of CB progenitors compared to BM progenitors at the time of infection.

**DISCUSSION**

Delayed platelet engraftment⁵,¹²,¹³ and graft failure⁶ have been reported in HCMV-infected patients after allogenic BMT. Furthermore, several studies have shown a suppressive effect of HCMV on the proliferation and differentiation of hematopoietic progenitors both in vivo and in vitro.¹⁵-²⁴

A limited number of patients eligible for allogenic BMT has an HLA-identical family donor. Therefore, considerable interest has arisen as to use CB as an alternative source of hematopoietic stem cells for allogenic transplantation. Several reports have shown that CB is a potent source of multipotent progenitors with ability to reconstitute hematopoiesis²⁵-³⁰,³⁶-³⁸ and recently CB cells have successfully been used in hematopoietic stem cell transplantation of children.³⁶,³⁷ To further examine the potential usefulness of CB as a source of hematopoietic progenitor cells in transplantation, we have studied the effect of HCMV on the growth and hematopoietic progenitor cells obtained from CB in comparison with the effect on BM progenitors.

In agreement with previous observations,¹⁶-²⁴ we have shown that a wild-type strain of HCMV highly suppress the HGF-induced growth of BM myeloid progenitors in colony assay. In contrast, the IL-3- and IL-3 plus SCF-induced progenitor growth of CB MNC were not inhibited, neither by this wild-type strain of HCMV nor by the laboratory strain AD169. This difference in susceptibility for HCMV between CB and BM progenitor cell growth was also observed using CD34⁺ cells in single cell assay.

Although HCMV did not have the ability to inhibit the proliferation of myeloid progenitors obtained from CB in vitro, we observed that HCMV was taken up by purified CD34⁺ CB cells in a proportion similar to that seen for CD34⁺ BM cells. Consistent with this, HCMV DNA was detected in both HCMV-infected CB and BM CD34⁺ cells and in day 14 colonies obtained from the soft agar assay. This indicates that the difference in the ability of HCMV to affect the growth of CB and BM myeloid progenitors could not solely be explained by a difference in the tropism of HCMV to BM and CB cells.

The binding and uptake of HCMV in permissive cells are followed by transcription of virus genes leading to production of HCMV specific regulatory proteins, followed by transcription and production of structural proteins and finally new virus.²⁹ We showed that HCMV could enter both CB and BM CD34⁺ progenitor cells, but we detected neither HCMV specific MIE transcripts nor IE1 and IE2 proteins in cultured CD34⁺ CB and BM cells. This is consistent with the findings of Minton et al⁴⁰ and Apperly et al⁴¹ but in contrast to the results reported both by Sing et al²² and Maciejewski et al²³, who showed that transcripts of HCMV MIE gene could be detected in cultured hematopoietic progenitor cells. These observed variabilities may be caused by variations in virus source, virus to cell ratio, or virus inoculation time used.²⁹

Although several studies have indicated that BM and CB progenitors may be comparable in many respects,²³,⁴¹ some studies have shown variabilities in the proliferative response of hematopoietic progenitors and their response to different inhibitors of hematopoiesis.³⁰,³²,³³ We found that CD34⁺ cells obtained from BM were 2.5-fold more cell cycle-active than CD34⁺ CB cells. This is consistent with results obtained by Lu et al⁴² using thymidine killing assay. The transcription of HCMV DNA is highly dependent of an active cell cycling status at the time of infection.³⁶ Thus, the slow cycling rate of naive CB progenitor cells at the time of infection could be a possible explanation for the observed resistance of these cells to HCMV in vitro. Therefore, we induced CD34⁺ CB progenitors to cycle with the hematopoietic growth factors, IL-3, SCF, and G-CSF for 24 hours before HCMV infection. This increased the proportion of CD34⁺ CB cells in S + G₂/M.

Several mechanisms have been postulated to explain how HCMV inhibits hematopoiesis in vitro. Some reports show that HCMV affects accessory cells by reducing their ability to support the hematopoiesis, whereas others indicate a direct effect of HCMV on hematopoietic progenitors that are abortively infected. The inhibitory effect of HCMV in these studies has been examined using colony forming or thymidine incorporation assays. In contrast to soft agar and thymidine incorporation assays, the single cell assay eliminates the indirect suppressive effect of HCMV on hematopoietic progenitor growth through accessory cells.

Consistent with the ability of a wild-type strain of HCMV to enter CD34 BM cells, we showed that HCMV inhibited the ability of CD34 BM progenitor cells to proliferate in single cell assays. Therefore, our results show that at least one HCMV strain has the ability to directly suppress proliferation of hematopoietic progenitor cells.

CB cells have been shown to be an alternative source of hematopoietic stem cells for allogeneic transplantation in children where HLA-matched BM family donors are not available. Furthermore, several in vitro studies have indicated that sufficient numbers of progenitors for transplantation of adults may be obtained by collection of 100 to 200 mL of CB. In addition, the immaturity of the CB T cells indicate that CB transplantation may lead to less graft-versus-host disease than unrelated HLA-matched BM transplants. Because morbidity and mortality related to HCMV infection are major obstacles in BMT, our results support the use of CB cells in allogeneic transplantation. However, further investigations are necessary to show that CB cells could have an advantage over BM cells for hematopoietic stem cell transplantation.

ACKNOWLEDGMENT
The authors want to thank Dynal for providing Dynabeads and DETACHaBEAD.

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Direct growth suppression of myeloid bone marrow progenitor cells but not cord blood progenitors by human cytomegalovirus in vitro

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