Apoptosis of CD4⁺ T lymphocytes in human herpesvirus-6 infection

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The present authors have recently reported that inoculation with human herpesvirus-6 (HHV-6) renders CD4⁺ T lymphocytes susceptible to apoptosis in vitro. In order to confirm that apoptosis of CD4⁺ T lymphocytes also occurs in HHV-6 infection in vivo, apoptosis of lymphocytes isolated from nine patients with exanthem subitum and from an adult patient with severe HHV-6 infection was examined. Peripheral blood mononuclear cells were cultured for 3 days and apoptosis of lymphocytes was then examined by flow cytometry of propidium iodide-stained DNA. The percentages of hypodiploid DNA, indicating apoptosis, in lymphocytes from 10 patients with HHV-6 infection were significantly higher than those from five infant patients with non-infectious diseases and five healthy adults (P < 0.0002). DNA fragmentation was also detected by agarose gel electrophoresis in lymphocytes from patients with HHV-6 infection. Apoptosis appeared to occur predominantly in CD4⁺ T lymphocytes and HHV-6 was isolated from the CD4⁺ T lymphocyte fraction. These data demonstrate that HHV-6 renders CD4⁺ T lymphocytes susceptible to apoptosis in vivo.

Human herpesvirus-6 (HHV-6) is a T-lymphotropic virus (Salahuddin et al., 1986; Takahashi et al., 1989) and the causal agent of exanthem subitum (Yamanishi et al., 1988). It has been reported that HHV-6 also causes various illnesses including hepatitis, pneumonitis, lymphadenitis, encephalitis and infectious mononucleosis-like disease, and the pathogenesis of these diseases has been a focus of interest. HHV-6 is also considered an important cofactor for the development of AIDS in individuals infected with human immunodeficiency virus 1 (HIV-1), based on several lines of evidence. Firstly, de novo expression of surface CD4 is induced in CD4⁻ T lymphocytes and natural killer cells following infection with HHV-6 (Lusso et al., 1991, 1993, 1995). Secondly, HHV-6 can coinfect CD4⁺ T lymphocytes with HIV-1, resulting in trans-activation of the HIV-1 long terminal repeat and acceleration of cell death (Ensoli et al., 1989; Lusso et al., 1989; Zhou et al., 1994). In addition to these findings, it has recently been demonstrated that inoculation with HHV-6 renders CD4⁺ T lymphocytes susceptible to apoptosis in vitro (Inoue et al., 1997). In order to determine whether apoptosis of CD4⁺ T lymphocytes also occurs in HHV-6 infection in vivo, apoptosis of peripheral blood lymphocytes isolated from patients with exanthem subitum and from an adult patient with severe HHV-6 infection was examined.

Peripheral blood mononuclear cells (PBMC) were isolated from patients and healthy individuals after obtaining their informed consent or that of their parents. PBMC were separated from infant patients who were diagnosed as having exanthem subitum on the basis of typical clinical manifestations and the isolation of HHV-6 from PBMC by coculture with cord blood mononuclear cells (Yamanishi et al., 1988) or detection of the HHV-6 genome in PBMC by PCR (Sada et al., 1996). PBMC were also separated from a 29-year-old patient with high fever, lymphadenopathy, generalized macropapular rash, renal failure and liver injury, who had been treated with cyclosporin A and sulfasalazine for psoriatic arthritis. The peripheral blood lymphocyte count of this patient was 1200 µl with a surface phenotype of CD3⁺ (62%), CD4⁺ (35%), CD8⁺ (30%) and CD25⁺ (25%). HHV-6 variant B was isolated from his PBMC using established methods (Yamanishi et al., 1988). The titre of anti-HHV-6 antibody, determined by indirect immunofluorescence on admission and 5 weeks after disease onset, was 160 and 5120, respectively. For the control group, PBMC were separated from five infant patients with noninfectious diseases and five healthy adults. The PBMC (2 × 10⁹) were suspended in 2 ml RPMI 1640 medium supplemented with 10% foetal calf serum and cultured in a 16-mm-diameter well. After 3 days, when the percentage of apoptosis was maximal, the cultured cells were harvested and the percentage of apoptotic cells was measured by flow cytometric analysis of DNA stained with propidium iodide (PI), as described previously (Nicoletti et al., 1991). DNA staining with PI in hypotonic buffer was performed using a CycleTest Plus DNA reagent kit (Becton-Dickinson), fol-
Fig. 1. Percentages of apoptotic cells in lymphocytes from patients with HHV-6 infection, patients with noninfectious diseases, and healthy individuals. ○, Patients with exanthem subitum; ●, adult patient with active HHV-6 infection; △, infant patients with noninfectious diseases; ▲, healthy adults.

following the manufacturer’s protocol. The PI fluorescence of individual nuclei was measured using a flow cytometer. Cell debris was gated out from the analysis by raising the light-scattering threshold. The percentage of apoptotic cells was determined by measuring the hypodiploid DNA peak. In some experiments, peripheral blood lymphocytes were separated into CD4⁺ and CD8⁺ fractions using monoclonal antibody-coated magnetic beads (Dynabeads, Dynal), following the manufacturer’s protocol, and used for the apoptosis analysis as described above.

DNA fragmentation into oligonucleosome units, which reflects apoptosis, was examined by agarose gel electrophoresis. Cells cultured for 3 days were collected from wells and centrifuged. The pellets were resuspended in 5 mM Tris–HCl pH 7.4 containing 0.5% SDS, 2 mM EDTA and 0.5 mg/ml proteinase K, and incubated for 1 h at 50 °C. RNase (50 µg) was then added and incubation at 50 °C was continued for 1 h. DNA was extracted from the resulting viscous solution with phenol–chloroform, precipitated with ethanol, dried and resuspended in TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The extracted DNAs were subjected to electrophoresis in 1.8% agarose gel and visualized by staining with ethidium bromide.

Fig. 1 shows the results of flow cytometric analysis of DNA fragmentation in cultured lymphocytes. Each value indicates the percentage hypodiploid DNA content of lymphocytes. Analysis by the Mann–Whitney U test showed that the percentages of hypodiploid DNA in lymphocytes of 10 patients with HHV-6 infection were significantly higher than those of the five patients with noninfectious diseases and five healthy adults (P < 0.0002).

Agarose gel electrophoresis of DNA from lymphocytes of a healthy individual, a patient with exanthem subitum, and an adult patient with HHV-6 infection, cultured for 3 days, is shown in Fig. 2. The ladder pattern of DNA on agarose gel electrophoresis, which reflects DNA fragmentation into oligonucleosome units, was scarcely detected in DNA from
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Fig. 3. Flow-cytometric DNA fluorescence profile. Peripheral blood whole lymphocytes of a healthy individual (A), a patient with exanthem subitum (B) and an adult patient with HHV-6 infection (C) were cultured for 3 days. CD4+ T lymphocytes (D) and CD8+ T lymphocytes (E) of an adult patient with HHV-6 infection were also cultured for 3 days. Nuclei were stained with PI and the DNA contents of the cells analysed by flow cytometry. The values above the bars are percentages of apoptotic nuclei.

lymphocytes of the healthy individual. On the other hand, DNA from lymphocytes of the patient with exanthem subitum and the adult with HHV-6 infection was markedly fragmented into multiples of oligonucleosome-length units.

Fig. 3 shows representative data from flow cytometric analysis of DNA stained with PI. Little increase in the nuclei with a hypodiploid DNA content was detected in lymphocytes of a healthy individual cultured for 3 days (Fig. 3A). On the other hand, the percentages of the hypodiploid DNA peaks in lymphocytes from a patient with exanthem subitum (Fig. 3B) and an adult patient with active HHV-6 infection (Fig. 3C) were increased after culture for 3 days. In order to clarify whether apoptosis occurs predominantly in CD4+ or CD8+ T lymphocytes, flow cytometric analysis of DNA was performed using fractionated lymphocytes. As shown in Fig. 3(D, E), apoptosis appeared to occur predominantly in CD4+ T lymphocytes from the adult patient with active HHV-6 infection. Flow cytometric analysis was also performed using CD4+ and CD8+ lymphocytes separated from an infant patient with exanthem subitum, and the same results (31.4% in CD4+ and 11.3% in CD8+ T lymphocytes) were obtained. HHV-6 was isolated from CD4+ lymphocytes but not from CD8+ lymphocytes, as described previously (Takahashi et al., 1989).

It has recently been reported that HHV-6 inoculation renders CD4+ T lymphocytes susceptible to apoptosis in vitro (Inoue et al., 1997). The present study was undertaken to clarify whether HHV-6 infection also induces apoptosis of CD4+ T lymphocytes in vivo. Our results showed that peripheral blood lymphocytes from the patients with exanthem subitum and the adult patient with severe HHV-6 reactivation underwent apoptosis without any stimulation. Although it has been reported that various viruses induce apoptosis in an in vitro experimental system, there are only a few papers describing apoptosis of human cells with natural virus infection (Shen & Shenk, 1995). The present results demonstrate that HHV-6 is indeed an inducer of apoptosis in CD4+ T lymphocytes in vivo.

In our previous study, it was found that apoptosis occurred predominantly in HHV-6 antigen-negative cells, and that HHV-6 virion-free supernatant of HHV-6-infected cells was able to induce susceptibility to apoptosis, suggesting that HHV-6 entry and replication are not necessarily required for induction of apoptosis in lymphocytes (Inoue et al., 1997). Although the percentage of cells in which HHV-6 replicates in the peripheral blood of individuals with HHV-6 infection is low (Takahashi et al., 1989), that of apoptotic cells after 3 days’ culture was as high as 20%. These results suggest that apoptosis in HHV-6 infection occurs predominantly in cells uninfected by HHV-6, as reported recently in HIV-1 infection (Finkel et al., 1995).

It is of considerable importance to clarify the lymphocyte subpopulation which undergoes apoptosis in HHV-6 infection. Since the age of most patients with exanthem subitum is less than 1 year, a sufficient amount of blood for analysis of apoptotic cell characteristics could be obtained from only two individuals with HHV-6 infection. Although the number of experiments was limited, flow cytometric analyses of lympho-
cytes from the two individuals clearly showed that apoptosis occurred predominantly in CD4+ lymphocytes. It has been reported that apoptosis occurs in cultured CD45RO+ primed T lymphocytes with both the CD4+ and CD8+ phenotype in Epstein–Barr virus (EBV)-induced infectious mononucleosis (Uehara et al., 1992). Peripheral blood T lymphocytes of patients with infectious mononucleosis are thought to be stimulated to proliferate by antigenic determinants of EBV. In the present study, apoptosis was detected predominantly in CD4+ T lymphocytes, and the number of peripheral blood lymphocytes in HHV-6 infection did not increase as much as that in EBV-induced infectious mononucleosis. In addition, in contrast to apoptosis in EBV infection, which peaked following 6 h incubation, apoptosis in HHV-6 infection needed a longer incubation period. These data suggest that the mechanisms of lymphocyte apoptosis in EBV and HHV-6 infection are distinct and that, in the case of HHV-6, activation of T lymphocytes in response to virus infection is not the major cause of apoptosis in CD4+ T lymphocytes. When considering the evidence that HHV-6 selectively infects CD4+ T lymphocytes in vivo (Takahashi et al., 1989), it is strongly suggested that apoptosis detected in peripheral blood lymphocytes of patients with HHV-6 infection is induced directly by HHV-6 infection. Although the precise mechanism of HHV-6-induced apoptosis is still obscure, it can be hypothesized that an HHV-6-derived component which has affinity for CD4, such as gp120 in HIV-1-induced apoptosis (Banda et al., 1992), and certain cytokines produced by HHV-6 infection, may render HHV-6-uninfected CD4+ T lymphocytes susceptible to apoptosis in vivo.

The significance of T-lymphocyte apoptosis in the pathogenesis of various HIV-6 infections is of considerable interest. An association of HHV-6 with histiocytic necrotizing lymphadenitis (Kikuchi’s disease) has been reported (Eizuru et al., 1989). It is well known that many apoptotic cells are present in affected lymph nodes of patients with Kikuchi’s disease, supporting the present finding that HHV-6 induces apoptosis of lymphocytes in vivo. It has been reported that HHV-6 is an important cofactor for the development of AIDS (Ensoli et al., 1989; Banda et al., 1992; Zou et al., 1994). Taken together with these previous reports, the present data demonstrating that HHV-6 itself induces apoptosis of CD4+ T lymphocytes via an innocent bystander mechanism, as reported recently for HIV-1-induced apoptosis (Finkel et al., 1995), provide new insight into the pathogenesis of AIDS. When considering the evidence that HHV-6 is frequently activated in AIDS patients (Corbellino et al., 1993; Knox & Carrigan, 1994), inhibition of HHV-6 activation may result in delayed development of AIDS in HIV-1-infected individuals. A report indicating that continuous administration of an anti-herpetic agent prolonged survival in HIV-1-seropositive persons (Stein et al., 1994) appears to support this hypothesis.

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References


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