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Major histocompatibility complex class I molecules are down-regulated at the cell surface by the K5 protein encoded by Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8

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The expression of major histocompatibility complex class I (MHC-I) molecules at the cell surface was down-regulated in BC-3 cells infected with Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus-8 at early times after treatment with 12-*O*-tetradecanoylphorbol acetate (TPA), and in HeLa cells transfected with the K5 gene of KSHV. However, an immunoprecipitation study on these cells with anti-MHC-I monoclonal antibody revealed that there was no significant reduction in the synthesis of MHC-I molecules. A pulse–chase analysis followed by endoglycosidase H digestion also demonstrated the stability and transport of MHC-I molecules from the endoplasmic reticulum to at least the medial-Golgi. K5 antigen was clearly detected by immunohistological examination of samples from Kaposi's sarcoma, primary effusion lymphoma and Castleman's disease. These results suggest that the down-regulation of MHC-I molecules by K5 gene expression during reactivation may be important for evading immunological surveillance in the host.

Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus-8 is a lymphotropic gammaherpesvirus related to other herpesviruses with oncogenic potential such as herpesvirus saimiri (HVS), Epstein–Barr virus (EBV) and the murine herpesvirus MHV-68 (Moore *et al.*, 1996). Sero-epidemiological studies have demonstrated that KSHV infection is tightly linked to KS risk (Gao *et al.*, 1996; Simpson *et al.*, 1996) and that this virus infection is associated with two other AIDS-related diseases, primary effusion lymphoma (PEL) (Cesarman *et al.*, 1995) and multicentric Castleman's disease (MCD) (Soulier *et al.*, 1995). Several cell lines have been established from patients with human immunodeficiency virus (HIV)-negative or -positive body cavity-based lymphoma, including BC-3 and BCBL-1 cells (Arvanitakis *et al.*, 1996; Renne *et al.*, 1996), and viral gene expression is highly restricted in these cells. Following treatment of these cells with sodium butyrate or 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a cascade of viral gene expression is induced, resulting in lytic replication of the virus (Miller *et al.*, 1996, 1997).

In general, viral gene products influence the normal expression of cellular genes. One such example of altered gene expression is the down-regulation of the major histocompatibility complex class I (MHC-I) at the cell surface. This has been well studied in cells that have been infected or transfected with adenovirus (Andersson *et al.*, 1985; Burgert & Kvist, 1985), herpes simplex virus (HSV) (Jennings *et al.*, 1985), human cytomegalovirus (HCMV) (Jones *et al.*, 1996; Lehner *et al.*, 1997; Wiertz *et al.*, 1996), murine cytomegalovirus (MCMV) (Campbell & Slater, 1994) and HIV (Schwartz *et al.*, 1996).

Our first attempt was to demonstrate the decrease of MHC-I expression in cells infected with KSHV as well as cells transfected with K5 gene. We previously reported that K5 is an endoplasmic reticulum (ER) resident protein (Haque *et al.*, 2000). Since ER resident proteins of other viruses have been shown to down-regulate MHC-I molecules, we designed experiments to examine whether the K5 gene product could down-regulate MHC-I molecules. BC-3 cells treated with TPA for 6, 12 and 24 h, and HeLa cells at 24 h post-transfection, were harvested and processed for FACS analysis with a fluorescent isothiocyanate (FITC)-labelled anti-HLA-A, B, C monoclonal antibody (MAb), W6/32 (Dako). Using MAb to K5 protein (Haque *et al.*, 2000), we first examined the cells by immunofluorescent antibody (IFA) test. As shown in Fig. 1(A), about 10–15% of the cells expressed the K5 protein by 6 h

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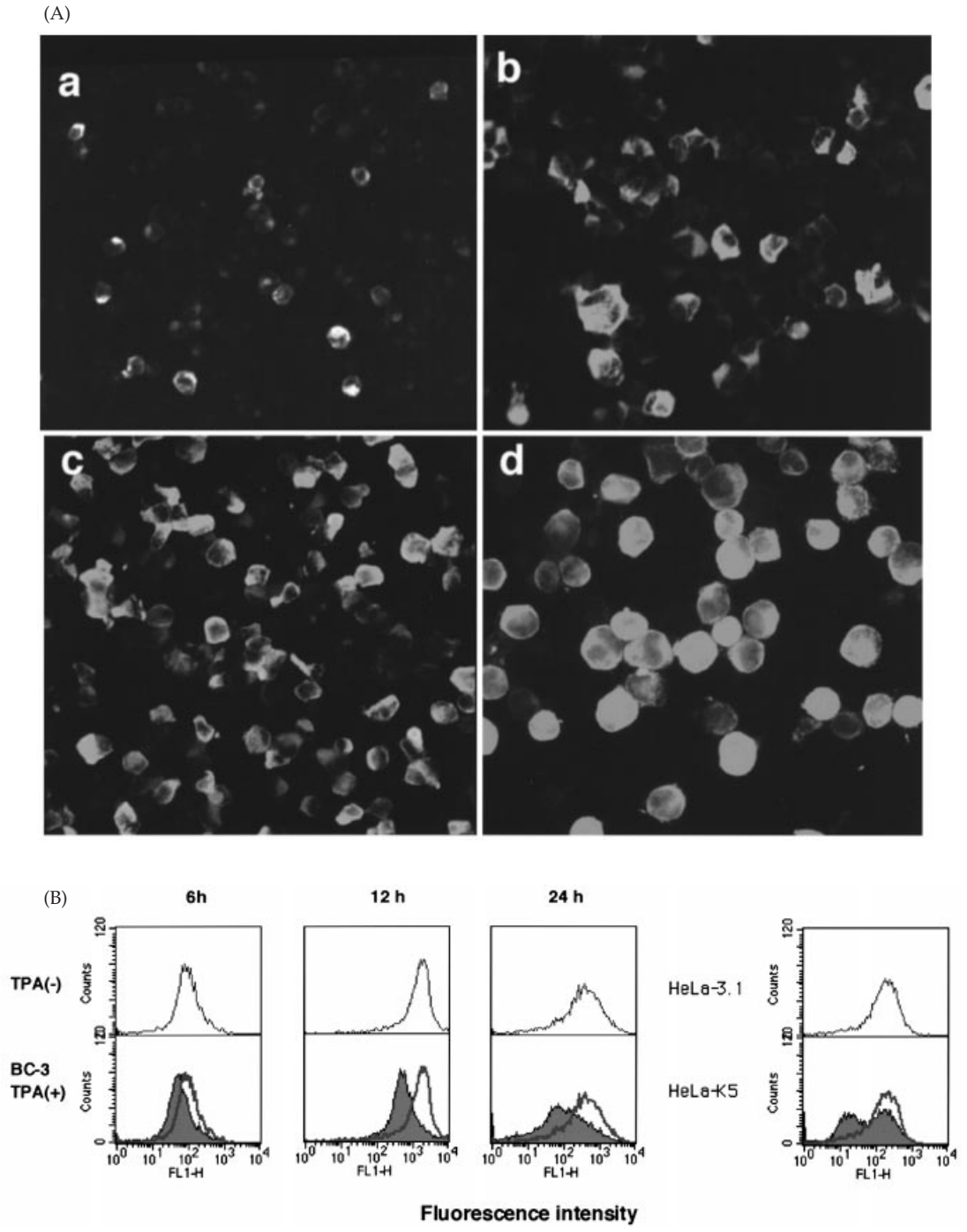

Fig. 1. Expression of K5 and the time-course of the down-regulation of MHC-I molecules. (A) Immunofluorescence analysis of TPA-treated BC-3 cells and K5-transfected HeLa cells at different times after induction or transfection, to evaluate the number of BC-3 cells entering lytic replication and the transfection efficiency of the HeLa cells using the anti-K5 MAb 328C7. Panels (a)–(c) represent the TPA-induced BC-3 cells at 6, 12 and 24 h respectively; (d) shows the K5-transfected HeLa cells at 24 h. (B) Detection of lowered cell-surface expression of MHC-I molecules by FACS analysis in BC-3 cells undergoing lytic replication and in HeLa cells expressing the K5 gene, at different times post-induction or -transfection, using an FITC-labelled anti-human MHC-I HLA-A, B, C MAb. Open areas in the upper row represent staining of control cells, and filled areas in the bottom row represent staining of TPA-treated and K5-transfected cells at the indicated times. (The experimental data in the bottom row are shown superimposed on the control data from the top row.)

after treatment with 20 ng/ml TPA (Fig. 1A, panel a). The percentage of positive cells increased to 40–50% by 12 h (Fig. 1A, panel b) and almost all cells were positive 24 h after treatment (Fig. 1A, panel c). We then used FACS analysis to examine the level of MHC-I expression on the cell surface. The peak with the highest relative intensity of fluorescence on the surface was observed with untreated BC-3 cells [Fig. 1B, BC-3 TPA(−)], but the peak shifted to low intensity with the TPA-treated BC-3 cells. The decrease of MHC-I on the cell surface of TPA-treated BC-3 cells was observed 6 h after treatment with TPA, and this decrease continued until 24 h in a kinetic fashion. When Ramos cells (a B-cell-derived cell-line used as a negative control) were treated with TPA, there was no shift of peak, as in the untreated sample (data not shown). These results indicated that some KSHV genes might be involved in down-regulation of MHC-I molecules. In order to identify a specific gene, we examined (by FACS analysis) HeLa cells that were transiently transfected with the K5 gene. The KSHV K5 gene fragment was amplified by PCR using primers with *Nhe*I and *Xho*I restriction sites at the 5′ and 3′ end respectively. The amplified fragment was digested with the enzyme, and then inserted into pcDNA3.1(+)/Zeo (Invitrogen) to create plasmid pcDNA3.1-K5. One day before transfection, HeLa cells (5×10^5) were seeded onto 60 mm dishes containing sterile glass coverslips and incubated at 37 °C in a 5% CO_2 incubator. They were transfected with the expression plasmid, pcDNA3.1-K5, using SuperFect Transfection Reagent (Qiagen), according to the manufacturer's instruction. HeLa cells were also transfected with a vector pcDNA3.1 as a negative control (HeLa-3.1 in Fig. 1B). At 24 h post-transfection, cells were washed with PBS, fixed with 2% paraformaldehyde for 20 min and permeabilized by 0·2% Triton-X 100 for 5 min; expression of K5 was then detected as described for IFA. When HeLa cells were stained with the MAb to K5 24 h after transfection, 60–70% of the cells expressed the K5 antigen (Fig. 1A, panel d), suggesting a relatively high transfection efficiency. Two peaks with low and high relative intensities of fluorescence were observed on the surface of HeLa-K5 cells, while only one peak, which had a high intensity of fluorescence, was observed on the untreated cells (Fig. 1B, right-hand panels). These findings indicated that the reduction of MHC-I expression on the cell surface was mediated by expression of the K5 gene and this effect was more prominent in the K5-transfected HeLa cells.

As a second approach to determine the mechanism of MHC-I down regulation by the K5 gene, the synthesis, stability and transport of MHC-I protein in TPA-treated BC-3 cells and K5-transfected HeLa cells were analysed. First, the effect of K5 expression on the synthesis of MHC-I molecules was evaluated using an immunoprecipitation assay. Both BC-3 cells treated with TPA and HeLa cells transfected with the K5 gene for 24 h were radiolabelled for 2 h with 110 μCi/ml [^{35}S]methionine and the cell lysates were immunoprecipitated with MAb W6/32, which reacts with the MHC-I complex, and analysed on 15% SDS–PAGE under reducing conditions. Untreated BC-3 cells and untransfected HeLa cells were used as controls. Neither the BC-3 cells treated with TPA nor the K5-transfected HeLa cells showed any significant change in the expression of MHC-I antigens, compared with their respective control cells, suggesting that equal amounts of MHC-I molecules were synthesized in both TPA-treated and untreated BC-3 cells, and in K5-transfected and control HeLa cells (Fig. 2A). Second, we examined the effects of lytic replication of KSHV on the stability of MHC-I antigens. TPA-treated or untreated BC-3 cells were radiolabelled with 400 μCi/ml [^{35}S]methionine for 15 min, and then chased for 30–120 min. After the chase period, the cells were lysed, incubated with anti-heavy chain antibody, and analysed on 15% SDS–PAGE as above. The results indicated that lytic replication of KSHV had no detectable effect on the stability of the MHC-I heavy chain (Fig. 2B). However, the density of a band from TPA-treated cells at 120 min chase was slightly fainter than that of the untreated sample (Fig. 2B). Coscoy & Ganem (2000) also reported that in pulse–chase experiments the half-life of MHC-I was reduced at later times in the presence of K5. Similar results were obtained in K5-expressing HeLa cells (data not shown). Then, we investigated the transport of MHC-I molecules in BC-3 cells treated with endo H. This enzyme cleaves immature, *N*-linked oligosaccharides residing in the ER and *cis*-Golgi – the more complex oligosaccharide forms in the medial-Golgi are resistant to endo H (Campbell & Slater, 1994). Lysates were prepared from BC-3 cells and immunoprecipitated with the anti-MHC-I MAb W6/32 as described above. The immunoprecipitates bound to protein G–Sepharose beads were divided into two equal aliquots in 50 mM potassium acetate buffer, pH 5·4, with 0·02% SDS and 0·1% NP-40, and heated to 95 °C for 5 min. After cooling on ice, 6 mU of endo H was added to one of the samples. Digestion was carried out for 8 h at 37 °C. The lysates were then fractionated by 10% denaturing SDS–PAGE and analysed by fluorography. The results shown in Fig. 2(C) demonstrate that MHC-I molecules in both untreated and treated BC-3 cells remained endo H-sensitive during the 15 min pulse-labelling and 30 min chase period, when compared with the samples that had not been exposed to endo H (compare lane 2 with 1; 4 with 3; 8 with 7; and 10 with 9) indicating that their location was inside the ER. However, after a 120 min chase, they acquired endo H resistance (Fig. 2C; compare lane 6 with 5, and 12 with 11), indicating their transport from the ER to the medial-Golgi. Identical results were demonstrated in K5-expressing HeLa cells (data not shown). Taken together, these results demonstrated that neither the lytic replication of KSHV nor the transfection of the K5 gene had a significant effect on the synthesis, stability or transport of MHC-I molecules.

Finally, we investigated expression of the K5 gene in clinical samples from KS, PEL and MCD patients by immunohistochemical staining of K5 antigen in biopsy or autopsy samples using MAb specific for K5 antigen as described

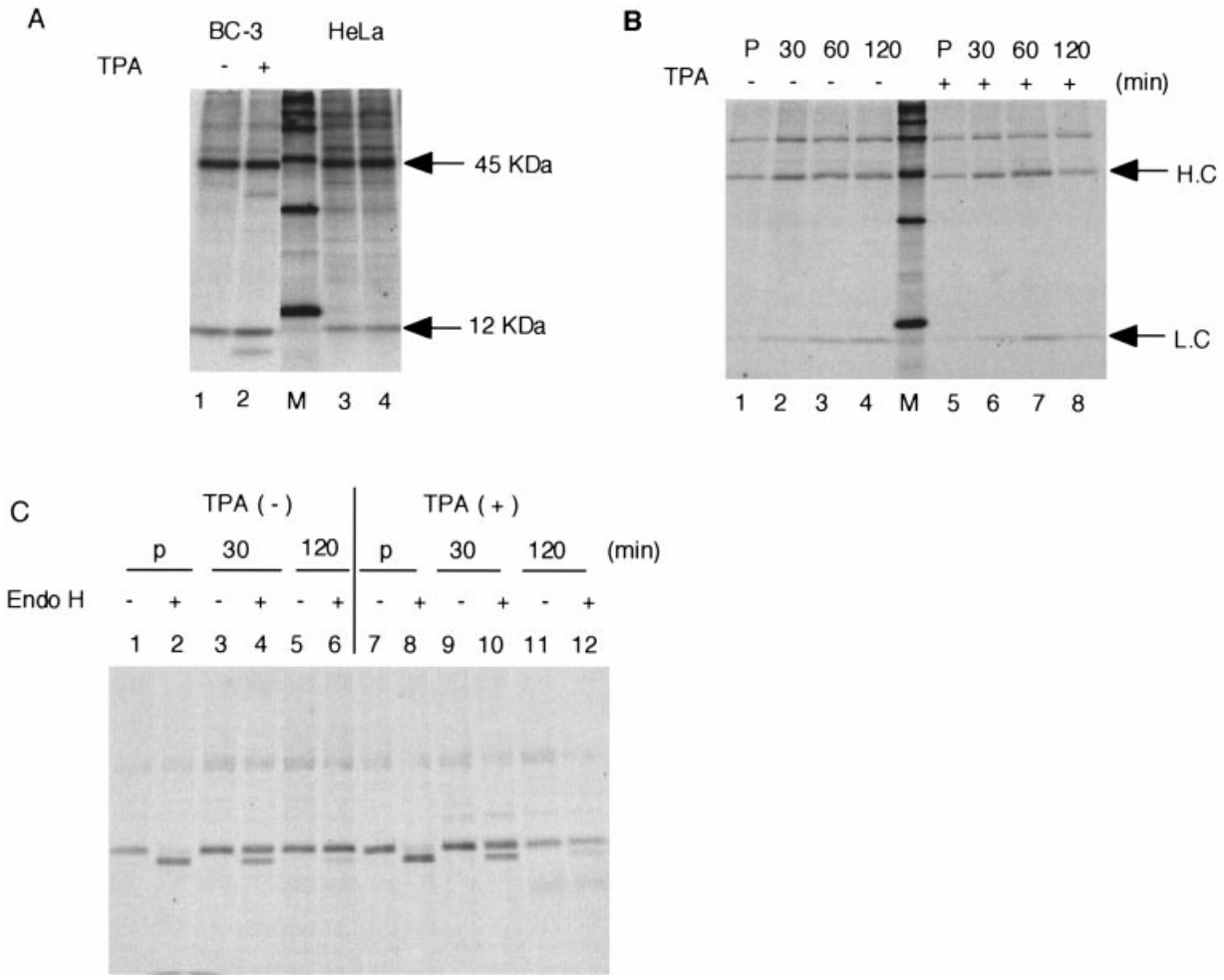

Fig. 2. Synthesis, stability and transport of MHC-I molecules. (A) Effect of KSHV infection and K5 transfection on biosynthesis of MHC-I antigens in BC-3 and HeLa cells. BC-3 cells were left untreated or treated with TPA (lanes 1 and 2, respectively). HeLa cells were transfected with empty vector or with K5-expressing vector (lanes 3 and 4, respectively). After 24 h the cells were metabolically radiolabelled for 2 h, immunoprecipitated with anti-MHC-I MAb W 6/32, and analysed by 15% SDS–PAGE. The 45 and 12 kDa bands represent the heavy and light chains, respectively. (B) Pulse–chase analysis shows the stability of MHC-I antigens in BC-3 cells. BC-3 cells were left untreated (lanes 1–4) or treated with TPA (lanes 5–8). After 24 h, the cells were metabolically radiolabelled for 15 min, and then chased for the indicated time (min) in unlabelled medium, immunoprecipitated with anti-MHC-I MAb W 6/32, and analysed by 15% SDS–PAGE. The positions of the heavy (H.C) and light chain (L.C) are indicated. (C) Endo H resistance of MHC-I molecules, demonstrating transport in BC-3 cells. BC-3 cells were left untreated or treated with TPA. At 24 h after induction, the cells were pulse-labelled for 15 min, and then chased for the indicated times (min) in unlabelled medium, immunoprecipitated with anti-MHC-I MAb W 6/32, and either left undigested (−) or digested with endo H (+) before being analysed by 10% denaturing SDS–PAGE.

elsewhere (Parravicini *et al.*, 2000). As shown in Fig. 3, K5 antigens were clearly detectable in cells from PEL (Fig. 3A), endothelial cells (Fig. 3B) and spindle cells (Fig. 3C) of KS, and cells in MCD (Fig. 3D).

Several mechanisms have been reported for the down-regulation of MHC-I molecules by virus infections, such as involvement of a requirement for assembly of a complex intracellular structure to support the efficient binding of peptides by MHC-I molecules. The components of this complex include TAP, tapasin, and the MHC-I heavy and light chains (Ploegh, 1998). Some viral gene products prevent the transport of MHC-I peptides from the ER by interfering with this complex (Ahn *et al.*, 1996; Burgert & Kvist, 1985; Jones *et al.*, 1996). In KSHV-infected cells, MHC-I molecules were synthesized normally and were stable (Fig. 2), suggesting that the K5 gene product does not prevent transport of MHC-I into the ER. Other possible mechanisms are that viral proteins bind to MHC-I molecules and retain them in the ER, and that viral proteins induce the retrograde-translocation of MHC-I molecules back into the cytosol. The E3/19K adenovirus 2 protein (Andersson *et al.*, 1985; Burgert & Kvist, 1985) and the US3 protein of HCMV (Ahn *et al.*, 1996; Jones *et al.*, 1996) have been shown to bind directly to MHC-I heavy chains, thereby preventing their transport to the cell surface. HCMV

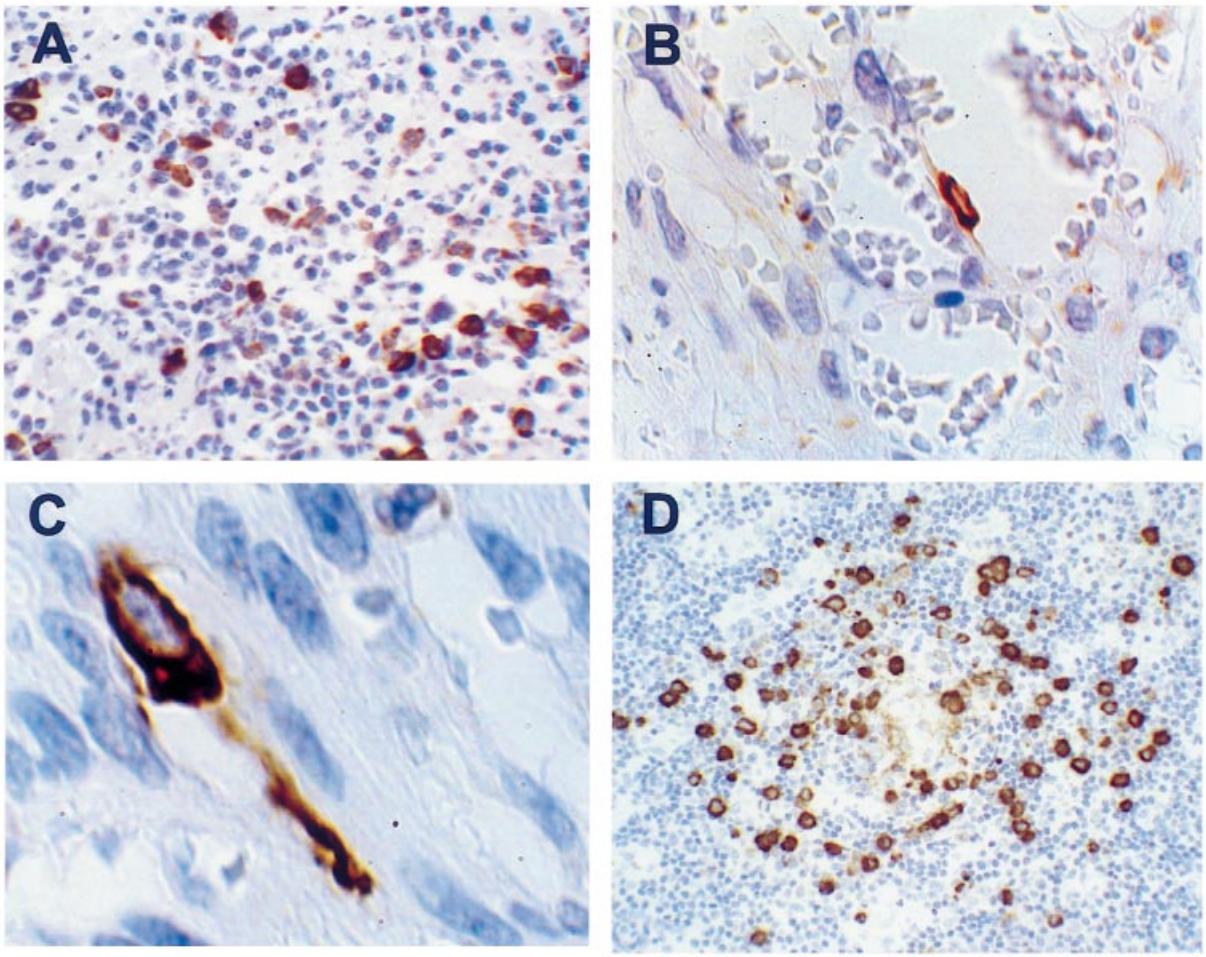

Fig. 3. Immunohistochemistry of K5 antigen in KS, PEL and MCD biopsy samples as detected by MAb to K5 antigen. (A) PEL specimen, (B) and (C) endothelial and spindle cells of KS specimens, respectively, as detected by the expression of K5, and (D) MCD.

US11 binds MHC-I molecules and redirects the MHC-I heavy chains to the cytosol (Wiertz *et al.*, 1996). We were unable to detect such an association between the K5 protein and the MHC-I heavy chain by immunoprecipitation analysis of K5-transfected HeLa cells or TPA-induced BC-3 cells, using either anti-K5 or anti-MHC-I MAbs. This binding association was detected using a mild detergent, such as digitonin (Ahn *et al.*, 1996; Jones *et al.*, 1996). However, even using 1% digitonin, we were unable to detect such an association (data not shown). Glycosylated MHC-I molecules were not retained in the ER and were processed to molecules that were resistant to endo H treatment, suggesting that MHC-I molecules were transported to the Golgi apparatus from the ER. A final possible mechanism is internalization of MHC-I molecules from the cell surface. The HIV Nef protein modifies the endocytic machinery and thereby down-regulates surface expression of the MHC-I complex (Schwartz *et al.*, 1996). K5-mediated down-regulation may be initiated by this mechanism, given that the MHC-I molecules were glycosylated and processed at least to the Golgi apparatus (Fig. 2C). Recently, similar results that support this idea have been reported (Ishido *et al.*, 2000; Coscoy & Ganem, 2000). These experiments were done in K3- and K5-transfected cell lines, but neither group reported MHC-I down-regulation in latently KSHV-infected cell lines. In our experiments, in addition to a K5-expressing cell line, we also demonstrated down-regulation of MHC-I molecules in latently KSHV-infected cells after TPA treatment.

It is interesting and important that such a phenomenon can be found *in vivo*. Results of immunohistochemical staining suggested that K5 antigen may be expressed in cells infected with KSHV during reactivation, and also may down-regulate MHC-I expression *in vivo*. In conclusion, we have demonstrated that the KSHV-encoded K5 protein down-regulates MHC-I molecules in KSHV-infected cells and have also detected expression of K5 antigen *in vivo*.

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