Induction of chemokine production by latent Kaposi’s sarcoma-associated herpesvirus infection of endothelial cells

Yiyiang Xu and Don Ganem

Infection with Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV) is linked strongly to development of KS, an endothelial neoplasm also characterized by striking neoangiogenesis and infiltration with inflammatory cells. To elucidate the links between endothelial infection and inflammation, primary human umbilical vein endothelial cells (HUVECs) were examined for the production of chemokines following latent KSHV infection. Several chemokines that are produced in the ground state, including MCP-1, NAP 2 and RANTES, are upregulated significantly by KSHV infection. Moreover, the chemokine CXCL16, which is nearly absent in uninfected cells, is induced significantly following infection. This induction is attributable primarily to expression of vFLIP, a known inducer of NF-κB. CXCL16 induces the chemotaxis of activated T cells, whose products have been proposed to positively regulate KS tumour-cell survival and growth. Whilst CXCL16 has also been proposed as a direct endothelial chemoattractant and mitogen, neither proliferation nor chemotaxis of HUVECs was observed following CXCL16 exposure. These results suggest that CXCL16 induction by KSHV contributes to the inflammatory phenotype of KS, but plays little role in the recruitment of endothelial spindle cells.

Kaposi’s sarcoma (KS) is a complex angioproliferative lesion linked strongly to infection with KS-associated herpesvirus (KSHV; also Human herpesvirus 8) (Schulz, 1999). KS is an unusual neoplasm that displays a very strong relationship to chronic inflammation, which it both induces and appears to depend upon (Ensoli & Stürzl, 1998; Ensoli et al., 2001). Clinically, KS lesions often progress during or following states of systemic inflammation, and KS tumours sometimes arise precisely at sites of antecedent local inflammation, such as surgical wounds (a property known to clinicians as the Koebner phenomenon) (Maral, 2000). Histologically, KS lesions display proliferation of spindle-shaped endothelial cells (which ultimately comprise the bulk of the tumour mass), but also dramatic neoangiogenesis and inflammatory infiltration. Considerable importance has been attached to the inflammatory component of KS, as: (i) in early KS, inflammatory changes predate the development of a detectable mass; (ii) KS lesions display abundant expression of proinflammatory cytokines (Ensoli & Stürzl, 1998); and (iii) KS cells in culture require conditioned medium from activated T cells for their survival and proliferation in vitro (Ensoli & Stürzl, 1998; Nakamura et al., 1988).

In KS tumours, KSHV infection is detected primarily in the endothelial-cell compartment of the lesion; most of these cells display latent KSHV infection, with only a small subset entering the lytic cycle (Boshoff et al., 1995; Staskus et al., 1999). KSHV latency has been studied most extensively in B cells, which are thought to be the primary reservoir of infection. Latently infected B cells are known to produce a variety of proinflammatory cytokines. Moreover, lytic induction of such cells is associated with the induction of several viral proteins with known proinflammatory properties, including a viral G protein-coupled receptor, a viral interleukin-6 (IL-6) homologue and several virally encoded chemokine homologues (Arvanitakis et al., 1997; Moore et al., 1996; Nicholas, 2005; Nicholas et al., 1997) (host IL-6 production is also induced strongly; Glaunsinger & Ganem, 2004). These molecules are likely to play important roles in several KSHV-related B-cell disorders, most notably multicentric Castleman’s disease, which is associated with pronounced lytic KSHV replication and striking IL-6 production (Oksenhendler et al., 2000; Parravicini et al., 2000; Staskus et al., 1999). Relatively less attention has been paid to the proinflammatory consequences of experimental endothelial infection by KSHV (although much has been written about the impact of KSHV infection on endothelial differentiation, proliferation and angiogenesis; Ciufo et al., 2001; Wang et al., 2004). Here, we have examined the impact of latent KSHV infection of primary vascular endothelial cells on the production and release of inflammatory mediators.
Primary human umbilical vein endothelial cells (HUVECs) were chosen for this study because of their ready availability and their susceptibility to KSHV infection. In fact, KSHV infection of such cells results in morphological changes typical of KS spindle cells \ vita - rearrangement of the actin cytoskeleton and cell elongation (Grossmann et al., 2006; Zhou et al., 2002). HUVECs were divided into two aliquots: one was mock-infected, whilst the other was infected with KSHV under conditions resulting in latent infection of virtually 100% of cells (as judged by expression of LANA protein visualized by indirect immunofluorescence). At 6 days post-infection, by which time the infected cells had undergone obvious morphological changes, culture supernatants were removed and assayed for chemokines by using a solid-phase immunoblotting procedure (RayBio Human Chemokine Antibody Array 1.1; RayBiotech) (Fig. 1a). The supernatants were applied to membranes containing antibodies to 38 human chemokines, each spotted in duplicate. Following binding, the membranes were washed and incubated with biotin-conjugated anti-chemokine antibodies, followed by incubation with horseradish peroxidase-conjugated streptavidin and development according to the instructions of the manufacturer. As shown in Fig. 1(b), the medium of uninfected HUVECs has considerable basal levels of several chemokines (including GRO, IL-8, MCP-1, MIP-1x, MIP-1y, eotaxin-3 and NAP 2). Following infection, several of these undergo significant upregulation – most notably MCP-1, NAP 2 and RANTES. Notably, one chemokine, CXCL16, was nearly undetectable in the basal medium, but induced strongly by infection. This induction was confirmed by quantitative ELISA (Fig. 1c).

To determine which viral latency gene influences CXCL16 expression most strongly, we transduced HUVECs with murine leukemia virus (MuLV)-based vectors driving expression of the individual KSHV latency loci – ORF 73 (LANA), ORF 72 (vCyclin), ORF 71 (vFLIP), ORF K12 (kaposin A), kaposin B or kaposin C. Four days following transduction and selection, the growth medium was assayed for CXCL16 by ELISA. As shown in Fig. 2(a), vFLIP expression upregulated CXCL16 expression strongly, whereas the other loci induced little (kaposin A) or no CXCL16 induction above the basal level. Examination of the vFLIP transductants for the levels of CXCL16 mRNA by quantitative RT-PCR revealed that the induction was at the

![Fig. 1. Latent KSHV infection induced CXCL16 secretion in HUVECs. HUVECs were infected latently with KSHV and conditioned medium was harvested for assay at 6 days post-infection (p.i.). (a) Map of antibodies to chemokines on the Raybio Human Chemokine Antibody Array 1.1. (b) Conditioned medium from latently KSHV-infected HUVECs or mock control was applied to the chemokine antibody arrays; bound chemokines were recognized by a pool of anti-chemokine antibodies corresponding to the antibodies spotted on the array. Arrows indicate spots of CXCL16 on the array. (c) CXCL16 ELISA was carried out with the conditioned medium.](http://vir.sgmjournals.org)
CXCL16 has an unusual biosynthetic scheme. It is made as a result of HUVECs infected authentically by KSHV (Fig. 2c), with a level of mRNA accumulation (Fig. 2b); the same is true of HUVECs infected authentically by KSHV (Fig. 2c), although the induction ratio is of lesser magnitude.

CXCL16 has an unusual biosynthetic scheme. It is made as a transmembrane protein (Matloubian et al., 2000) and cleavage of this form by the protease ADAM10 in its ectodomain leads to release of the extracellular ligand (Ludwig et al., 2005). To see whether upregulation of ADAM10 might contribute to enhanced expression of soluble CXCL16, we assayed levels of ADAM10 mRNA by quantitative RT-PCR in both KSHV-infected and vFLIP-transduced HUVECs. In neither case was ADAM10 mRNA induced (see Supplementary Fig. S1, available in JGV Online); although we cannot exclude a component of post-transcriptional regulation of ADAM10 activity, the induction of CXCL16 mRNA by vFLIP would appear to be sufficient to explain the upregulation of the secreted chemokine.

vFLIP’s primary biochemical activity is the induction of NF-κB activation via stimulation of IKK activity (Chaudhary et al., 1999; Field et al., 2003; Liu et al., 2002; Matta & Chaudhary, 2004), although other signalling activities of vFLIP have been reported (An et al., 2003). To validate that the upregulation of CXCL16 was dependent upon NF-κB activation, we tested the ability of a clone expressing an IκB super-repressor (IκB-SR) to block induction of the chemokine (IκB-SR lacks sites for phosphorylation by IKK, and hence cannot release active NF-κB from IκB after IKK stimulation). HUVECs were transduced with a murine retroviral vector expressing IκB-SR (or with the empty vector only). After 48 h, the cells were then transduced with the vFLIP-expressing retrovirus; 4 days later, conditioned medium was harvested and assayed for CXCL16 production by ELISA. Fig. 3(a) shows that expression of IκB-SR reduces induction of CXCL16 substantially, whereas infection with the empty vector had no effect.

To determine the cell-type specificity of CXCL16 induction, we examined a panel of immortalized, established cell lines of many different types for their ability to express CXCL16 in response to KSHV infection (Fig. 3b) or vFLIP transduction by MuLV-based vectors (Fig. 3c). CXCL16 induction was not observed in any of the established cell lines that we tested, following either viral infection or vFLIP overexpression; this survey included immortalized endothelial lines (SLK and TIME), as well as transformed cells of epithelial (RPE) and fibroblastic (MCF10A) lineage. To date, we have observed vFLIP induction of CXCL16 only in differentiated primary endothelial cells. Consistent with this, vFLIP transduction into primary microvascular endothelial cells of blood (BEC) or lymphatic (LEC) lineage also displayed CXCL16 induction (data not shown).

As most cells expressing vFLIP display NF-κB induction (An et al., 2003; Chaudhary et al., 1999; Matta & Chaudhary, 2004; Sun et al., 2003a, b, 2005, 2006), it seems clear that the ability to upregulate CXCL16 mRNA must be controlled by additional factors. Most likely, HUVECs are permissive for a post-NF-κB regulatory step that most lines do not support. Possibilities for such steps include transcript elongation, termination, stabilization or export. Alternatively, non-responsive cells might harbour repressive chromatin structures at this locus that prevent transcription induction by NF-κB.

CXCL16 is a recently identified chemokine first identified in mice (Matloubian et al., 2000). There, it is associated with chemotaxis of activated (but not resting) T cells in vivo and in vitro. More recent in vitro studies have suggested that the protein can trigger chemotaxis of endothelial cells (HUVECs) in vitro (Zhuge et al., 2005). As such activities could be very germane to KS pathogenesis in vivo, we deemed it important to verify them independently. Accordingly, HUVECs were placed in the upper well of a Transwell chamber (Corning) and recombinant CXCL16 was added to the lower well of the chamber; cells that migrated across the chamber filter toward the lower surface were counted after 3 h incubation at 37°C.
However, no significant chemotaxis was observed, even though chemotaxis to a known endothelial attractant, bFGF, was unimpaired (see Supplementary Fig. S2, available in JGV Online). We also examined whether HUVECs can be stimulated to proliferate following exposure to the chemokine, as judged by BrdU incorporation. As shown in Supplementary Fig. S3 (available in JGV Online), CXCL16 did not stimulate BrdU incorporation into HUVECs, whereas known endothelial mitogens such as bFGF were highly active in this assay.

These data indicate that viral infection of differentiated primary endothelial cells can trigger a programme of proinflammatory gene expression that differs somewhat from that observed in non-endothelial cells and includes the induction of the unusual chemokine CXCL16. The principal KSHV determinant of CXCL16 production appears to be expression of vFLIP, although lesser contributions by other viral latency proteins may occur. Upregulation by vFLIP appears to be primarily via NF-κB-mediated induction of CXCL16 mRNA production. The fact that CXCL16 induces migration of activated T cells is of interest as, in cell-culture systems, KS cells appear to require conditioned medium from activated T cells for survival and proliferation. It is therefore possible that CXCL16 induction may play an indirect, paracrine role in promoting survival and expansion of the tumour. A direct role for CXCL16 in endothelial chemotaxis and growth has also been postulated by others (Zhuge et al., 2005), but we cannot confirm their observations and think it likely that the protein does not itself stimulate endothelial migration or proliferation directly. Finally, we note that the induction of CXCL16 occurs only in primary endothelial cells and not in established lines of other lineages (even immortalized endothelial cells do not display this behaviour). This re-emphasizes the need to use cells as close as possible to those targeted naturally for infection when examining host–viral interactions designed to model one or more aspects of virus-induced disease.

**References**


