Short Communication

Expression of a Virus-Derived Cytokine, KSHV vIL-6, in HIV-Seronegative Castleman's Disease

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**Castleman's disease is a rare B cell lymphoprolifera-
tive disorder related to excess interleukin-6 (IL-6)-like
activity. Kaposi's sarcoma-associated herpesvirus
(KSHV or HHV8), which encodes a functional cyto-
kine (vIL-6), has been found in some patients with
Castleman's disease. Lymph nodes from 14 HIV-sero-
negative Castleman's disease patients were compared
to hyperplastic lymph nodes from 25 HIV-seronega-
tive patients as well as Kaposi's sarcoma lesions from
48 patients for KSHV infection and vIL-6, human IL-6,
and Epstein-Barr virus EBER expression. While all
Kaposi's sarcoma tissues examined were polymerase
chain reaction-positive and all control lymph nodes
were polymerase chain reaction-negative for KSHV,
none had detectable vIL-6 expression. Six of 14 (43%)
Castleman's tissues were positive for KSHV by poly-
merase chain reaction and all 6 had evidence of vIL-6
expression by immunohistochemistry. vIL-6-positive
Castleman's disease patients generally had the multi-
centric plasma cell variant form of the disease and
had a rapidly fatal clinical course frequently associ-
ated with autoimmune hemolytic anemia and gam-
mopathy. In contrast, 7 (88%) of the 8 vIL-6-negative
Castleman's disease patients had localized disease and
have remained disease-free after therapy. KSHV vIL-6
expression appears to be limited to hematopoietic
cells and is not present in Kaposi's sarcoma spindle
cells. These data suggest that Castleman's disease is a
syndrome of multiple etiologies involving aberrant
IL-6 activity from either endogenous or viral sources.
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Castleman's disease is an uncommon, non-neoplastic, lymphoproliferative disorder associated with an increased risk for developing Kaposi's sarcoma (KS) and lymphoid malignancies.[1,2] Castleman's disease can be histopathologically subdivided into hyaline vascular, plasma cell, or mixed variants based on degree of capillary proliferation, follicular involution, and plasmacytic infiltration in affected lymph nodes.[3,4] While hyaline vascular Castleman's disease is usually localized and treatable by surgical resection, plasma cell variant Castleman's disease frequently presents as a multicentric lymphadenopathic condition accompanied by systemic symptoms and multiple laboratory abnormalities.

Interleukin 6 (IL-6) is a cytokine which enhances B cell survival and proliferation,[5,6] and IL-6 overexpression has been pathogenetically linked to Castleman's disease.[7–10] Castleman's disease patients often have excess IL-6 production within lesions, and *in situ* IL-6 overexpression in mouse hematopoietic cells through use of a retroviral vector leads to a polyclonal hypergammaglobulinemia with plasma cell hyperplasia mimicking human Castleman's disease.[11,12] Further, treatment of Castleman's disease with a monoclonal antibody against IL-6 has been reported to have therapeutic benefit.[13] Clinical features and secondary diseases such as hemolytic autoimmune anemia and gammopathies associated with Castleman's disease are likely to result from polyclonal lymphocytic expansion due to IL-6 or IL-6-like overactivity.

Castleman's disease may occur among HIV-seropositive persons where it is a diagnosis of exclusion. Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) is a newly described gammaherpesvirus likely to cause KS,[14] which encodes several unique cytokines including a functionally active IL-6-like viral protein (vIL-6).[15,16] Nearly all HIV-seropositive patients and approximately one-half of HIV-seronegative patients with multicentric Castleman's disease are infected with KSHV.[17–19] HIV-

seropositive Castleman's disease patients have higher KSHV viral loads in affected tissues than do HIV-seroneg-ative/KSHV-positive patients.[17] While vIL-6 is similar to human IL-6 (huIL-6) in its biological activity,[15,20] it may have a broader tissue trophism due to its ability to directly bind and activate the gp130 signal transduction pro-tein.[21] Since KSHV infection is common among HIV-pos-itive patients regardless of the presence or absence of Castleman's disease,[22,23] we examined vIL-6 expression by immunohistochemistry among HIV-seronegative Castleman's disease patients.

Materials and Methods

Castleman's Disease Patients and Controls

Fourteen lymph node specimens from Italian HIV-sero-negative Castleman's disease patients were examined histopathologically and assigned to hyaline vascular, plasma cell, or mixed subtypes based on criteria pub-lished by Castleman[24] with subsequent modifications.[3,4] A cytospin of peripheral blood mononuclear cells was obtained from patient 1 for immunohistochemistry. For two Castleman's disease patients who developed non-Hodgkin's lymphoma, paraffin sections of the lymphoma were also examined. Twenty-five HIV-seronegative pa-tients with non-neoplastic reactive lymph nodes showing varying degrees of follicular hyperplasia were also inves-tigated. Control patients included 14 patients with breast, colon, or gastric cancer, 1 patient with hyperplastic lymphadenitis with Warthin-Finkeldey cells, 5 patients with toxoplasmosis, 3 patients with infectious mononucle-osis, 1 patient with syphilis, and 1 patient with cat scratch disease. None of the control patients had Kaposi's sar-coma, lymphoma, or microscopic evidence of tumor me-tastasis. Cutaneous KS biopsies included 13 lesions from HIV-seronegative patients and 35 lesions from HIV-sero-positive patients.

Formalin-fixed paraffin-embedded pellets of the KSHV-infected cell lines BC-1, BCBL-1, and BCP-1,[22,25,26] ei-ther unstimulated or after stimulation with 20 ng/ml 12-O-tetradecanoylphorbol-13-acetate were used as positive controls for immunohistochemistry.[15] P3HR-1, an Ep-stein-Barr virus (EBV)-positive, KSHV-negative cell line, was prepared in the same manner and used as negative control.

Immunohistochemical Analysis

Lymphocyte subpopulations, monocytes, and follicular dendritic cells were stained with antibodies against CD45RO (clone UCHL1), CD20 (clone L26), CD68 (Mac 367), DRC (clone DRC1), EMA (clone E29), CD30 (clone K1), and rabbit antisera against CD3, human IgD, and human IgM, all obtained from Dako (Glostrup, Denmark). Plasma cells were stained with BB4 mouse monoclonal antibody against CD138 (Sixth Workshop on Human Leu-kocyte Antigens, Osaka, Japan, November 1996). Anti-body binding was revealed using peroxidase-labeled rabbit anti-mouse or swine anti-rabbit antisera (Dako)

followed by TSA amplification (DuPont/NEN, Boston, MA). Expression of KSHV vIL-6 was evaluated using a polyclonal rabbit antiserum raised against vIL-6 peptides that does not cross-react with human IL-6 by Western blotting or immunohistochemistry.[15] Preimmune rabbit serum was used as negative control. Human IL-6 immu-nostaining[8] was performed on frozen sections only, using a specific monoclonal antibody (clone 4006) obtained after immunization with the human cytokine (kindly pro-vided by J. Van Damme, Rega Institute, Leuven, Bel-gium). Reactions were developed using diaminobenzi-dine (Sigma, St. Louis, MO) or aminoethyl carbazole (Dako) as chromogenic substrates, and sections were counterstained with hematoxylin. For double immuno-staining, the second primary antibody was revealed us-ing alkaline phosphatase-conjugated antisera and Fast Blue (Sigma) as chromogen.

Detection of KSHV and EBV Infection

Results of polymerase chain reaction (PCR) analysis for KSHV sequences on 6 of the 14 Castleman's disease biopsies has been previously reported.[19] The same set of primers, probes, and amplification conditions were used to analyze the remaining 8 Castleman's disease cases, KS tissues, and control lymph nodes. DNA was extracted from formalin-fixed paraffin-embedded tissue sections according to the method reported by Shimuzu et al.[27] EBV-infected cells were detected by in situ hybridization using a commercially available mixture of fluorescein-conjugated riboprobes specific for EBV-EBER RNAs (Dako) followed by an anti-fluorescein mouse monoclonal antibody (Dako).

Results

Expression and Cell Localization of KSHV vIL-6

Of the 14 patients with histologically confirmed Castle-man's disease, 11 had a plasma cell variant or mixed plasma cell-hyaline vascular morphology characterized by large sheets of interfollicular BB4-positive mature plasma cells and hyperplastic follicles with large germi-nal centers. The remaining three samples had the hyaline vascular morphology composed of hyaline atrophic ger-minal centers with radially penetrating vessels and prom-inent capillary proliferation in paracortical areas. Six (55%) of the 11 plasma cell/mixed variant tissues and none of the 3 hyaline vascular variant tissues were pos-itive for KSHV infection by PCR.

The six KSHV PCR-positive samples all contained cells with prominent cytoplasmic vIL-6 staining by immunohis-tochemistry (Figure 1A). KSHV vIL-6-positive cells were present in both hyperplastic and hyaline germinal cen-ters, but their numbers were highly variable in different areas of the same lymph node, ranging from 2 to 13% of the cells in the mantle zone (Figure 1B). KSHV vIL-6 expression was generally restricted to a subset of cen-troblastic/immunoblastic cells present among the mantle zone lymphocytes or, more rarely, located at the periph-
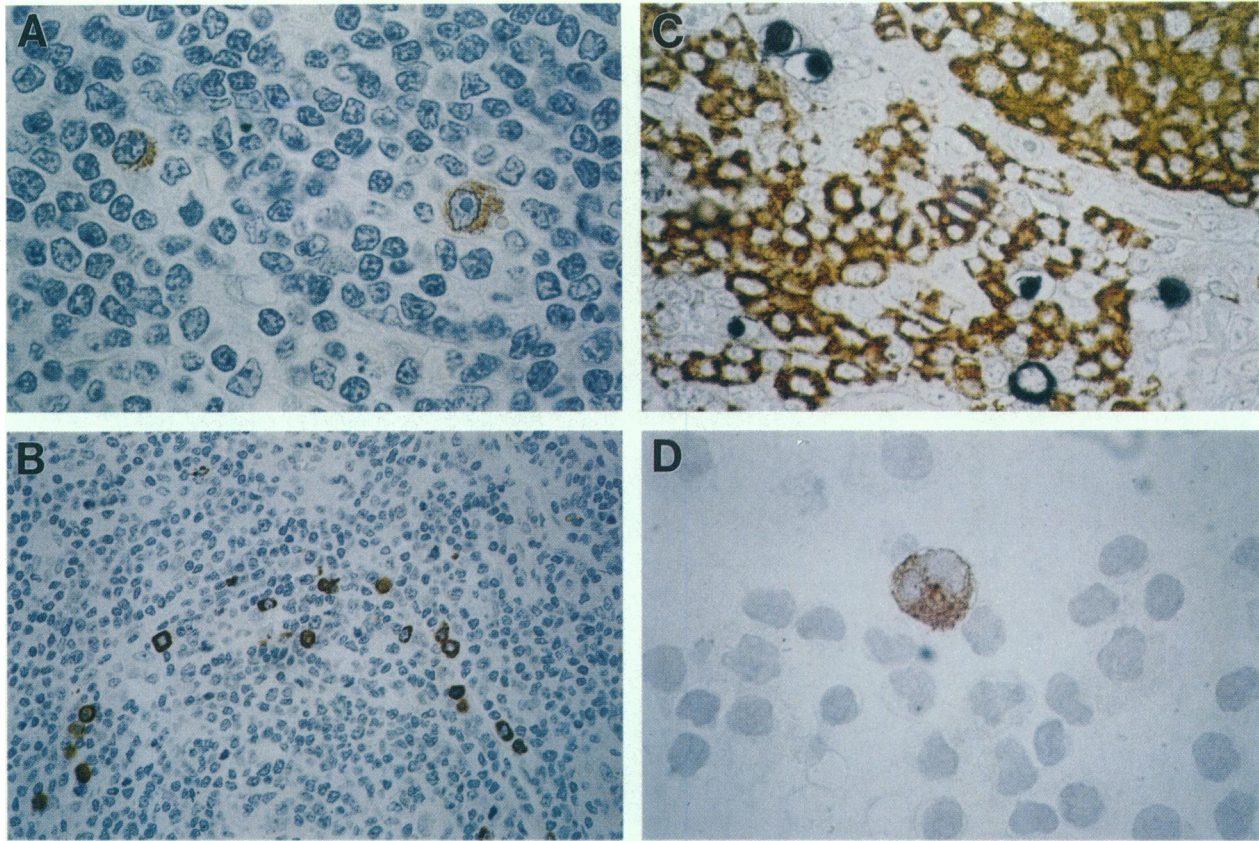
Figure 1. A: Plasma cell variant Castleman's disease lymph node demonstrates KSHV vIL-6 expression (diaminobenzidine; **brown**) localized to the cytoplasmic compartment. Positive cells frequently have centroblastic/immunoblastic appearance with large eccentrically placed nuclei. B: KSHV vIL-6-positive cells arrayed around periphery of germinal center in plasma cell variant Castleman's disease lymph node. C: Double labeling for KSHV vIL-6 (**black**) and CD20 (**brown**) show that KSHV-infected cells in Castleman's disease do not express this B cell marker. D: Cytospin preparation of peripheral blood from Castleman's disease patient with rare KSHV-infected circulating cells expressing vIL-6.

ery of germinal centers. In addition, vIL-6-positive immunoblastic cells were found randomly dispersed within poorly defined nodular aggregates of CD20⁺ B cells occasionally present in the paracortical areas. vIL-6-positive cells were nonreactive for CD20 (Figure 1C), CD138, CD45RO, CD3, CD68, CD30, and EMA. Rare peripheral blood cells from a cytospin preparation from patient 1 demonstrated vIL-6 expression (Figure 1D).

EBV EBER hybridization was detected in two plasma cell variant Castleman's disease biopsies and in five of the control lymph nodes, including all three cases of infectious mononucleosis. Both of the EBV-positive Castleman's disease biopsies were from KSHV-infected patients. In these cases, only a few cells positive for EBER transcripts were present and were localized to paracortical areas, whereas vIL-6-positive cells were present in the mantle zone. There was no evidence of EBV EBER and KSHV vIL-6 colocalization to the same cells in these patients. In comparison to vIL-6 (Figure 2, A and C), expression of huIL-6 was detectable in both plasma cell variant (4 cases examined; Figure 2B) and hyaline vascular variant (2 cases examined; Figure 2D) Castleman's disease tissues that were available as frozen sections. In these cases, most of the germinal centers were positive, showing staining localized to the follicular dendritic cells. In addition, rare cells randomly dispersed

in the paracortical areas were also positive. Reactivity for huIL-6, however, was also observed in 6 of 17 (35%) of hyperplastic lymph nodes from control patients without Castleman's disease in the same staining pattern as seen for huIL-6 in Castleman's disease lymph nodes.

Clinical Course of Disease

Clinicopathological findings for the Castleman's patients are reported in Table 1. At the time of diagnosis, all 6 KSHV-infected Castleman's disease patients (median age 62.5 years, range 57–81 years) had multicentric lymphadenopathy with systemic symptoms. Severe auto-immune hemolytic anemia or Kaposi's sarcoma developed in 3 and 4 of the 6 patients, respectively. Symptoms were rapidly progressive in the KSHV-positive Castleman's disease patients; 5 (83%) of 6 died 1 to 6 months (median 3 months) after diagnosis.

Of the 5 patients with KSHV-negative plasma cell Castleman's disease (median age 40 years, range 12–52 years), 4 presented with localized lymphadenopathy and systemic symptoms. One had high grade non-Hodgkin's lymphoma diagnosed 1 year before Castleman's disease onset, another had a clinical history of renal amyloidosis, and a third patient developed systemic lupus erythema-
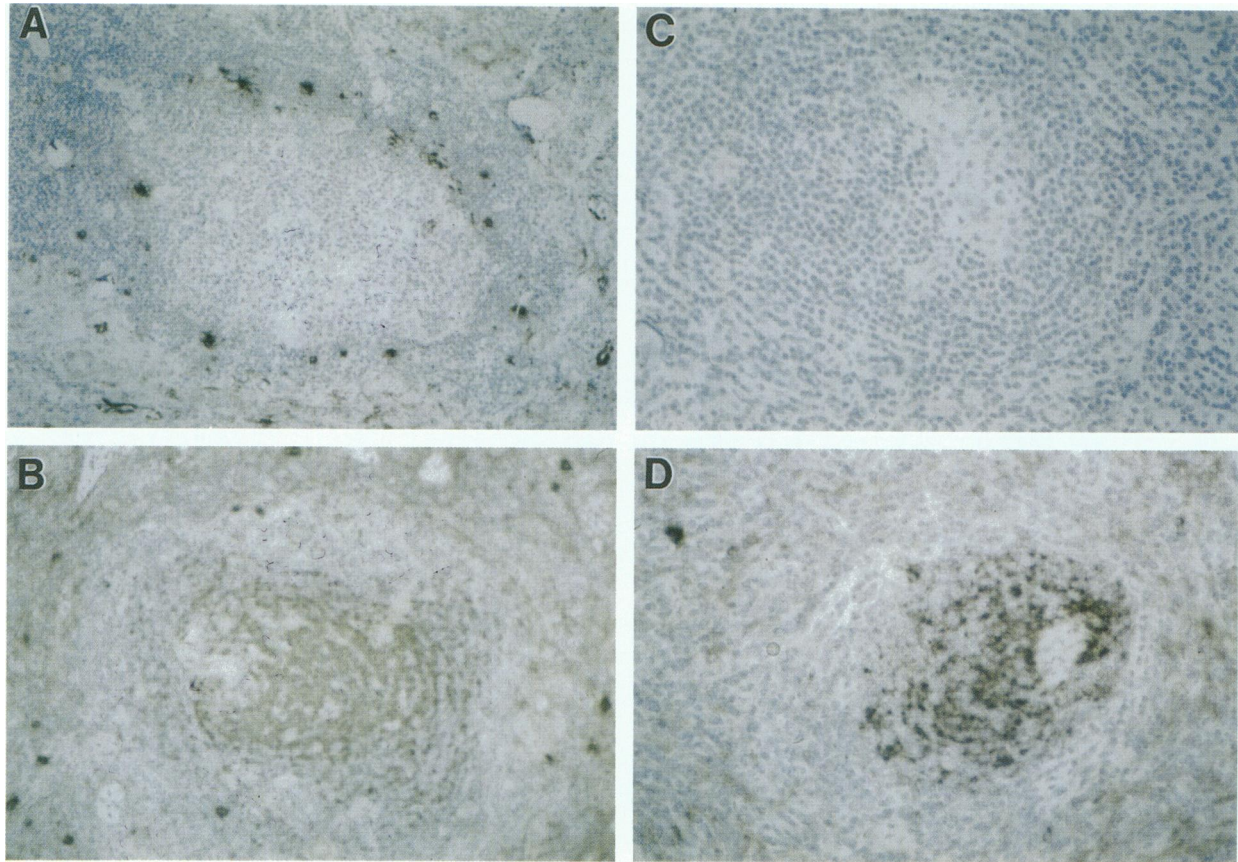
Figure 2. A: Frozen-section lymph node biopsy of plasma cell variant Castleman's disease shows scattered KSHV vIL-6-positive cells cuffing germinal centers. **B:** huIL-6 expression in the same lymph node found predominantly in germinal centers and in rare surrounding cells. **C:** Hyaline vascular variant Castleman's disease lymph nodes do not express KSHV vIL-6 by immunohistochemistry, which correlates with lack of PCR detection for KSHV DNA. **D:** KSHV-negative hyaline vascular variant Castleman's disease shows distribution of huIL-6 expression in germinal centers similar to plasma cell variant.

tosus 4 years after Castleman's disease diagnosis. These patients have remained free of Castleman's disease for 10 to 132 months after diagnosis and therapy (mean follow-up 65 months). Similarly, the three KSHV-negative, hyaline vascular Castleman's disease patients re-

sponded well to localized therapy, one of whom developed a high grade non-Hodgkin's B cell lymphoma 18 months after initial Castleman's disease diagnosis which was negative for both EBV EBER and KSHV vIL-6 expression.

Table 1. Clinical/Pathological Features of Patients with Castleman's Disease

Case No.	Histo-logical variant	KSHV v-IL6	KSHV PCR	EBV EBER	Age	Sex	Multicentric lymphade-nopathy	Type B symptoms	Clinical presentation	Intercurrent disease	Follow-up (months)	Out-come
1	PC+KS	Pos.	Pos.	Neg.	57	M	Yes	Yes	AHA	Multiorgan failure	1	Death
2	PC (mix)	Pos.	Pos.	Neg.	72	M	Yes	Yes	AHA		3	Death
3	PC (mix)	Pos.	Pos.	Pos.	60	M	Yes	Yes	Hyper G+KS	KS	6	Death
4	PC (mix)	Pos.	Pos.	Pos.	65	M	Yes	Yes	AHA	KS	6	Death
5	PC (mix)	Pos.	Pos.	Neg.	81	F	Yes	Yes	MC IgG lambda	Bronchopneumonia	1	Death
6	PC	Pos.	Pos.	Neg.	26	F	Yes	Yes	Kidney transplant	KS	8	Alive
7	PC (mix)	Neg.	Neg.	Neg.	15	M	No (mesent.)	Yes	Hyper IgG		64	Alive
8	PC (mix)	Neg.	Neg.	Neg.	40	M	No	No	MC IgG lambda	MC IgG lambda	68	Alive
9	PC (mix)	Neg.	Neg.	Neg.	38	F	Yes	Yes	Anti-nuclear antibodies	Systemic lupus erythematosus	132	Alive
10	PC	Neg.	Neg.	Neg.	52	M	No	Yes	Renal amyloidosis	Renal amyloidosis	10	Alive
11	PC	Neg.	Neg.	Pos.	40	F	No	Yes	NHL	NHL	40	Alive
12	HV	Neg.	Neg.	Neg.	15	F	No (retrop.)	No	Recurrent infections		Lost	Lost
13	HV	Neg.	Neg.	Neg.	18	F	No	No			66	Alive
14	HV	Neg.	Neg.	Neg.	58	F	No (retrop.)	Yes		NHL	50	Alive

PC, plasma cell type; HV, hyaline vascular type; NA, not available; KS, Kaposi's sarcoma; AHA, autoimmune hemolytic anemia; NHL, non-Hodgkin's lymphoma; MC IgG, monoclonal IgG.

Discussion

Our study suggests that Castleman's disease in HIV-negative patients is a heterogeneous disorder despite pathological similarities among various forms of the disease. Histopathological features of multicentric Castleman's disease (hyperplastic follicles with hyaline formation, plasmacytosis, and vascular hyperplasia) are nonspecific and have been reported to occur in a variety of other disorders such as lymphomas, autoimmune diseases, and some carcinomas. The diagnosis of Castleman's disease is frequently one of exclusion after careful screening for alternative diseases which are able to induce Castleman's disease-like lymph node changes.[2]

Detection of vIL-6 expression in KSHV-associated Castleman's disease lends support to IL-6 or IL-6-like overactivity being a central feature of this lymphoproliferative disorder. In our series, KSHV-infected, vIL-6-expressing lesions were more likely to have a multicentric plasma-cell morphology which was rapidly fatal. Three of the six KSHV-infected patients had autoimmune hemolytic anemia, and two had mono-/polyclonal gammapathy consistent with abnormal proliferation of plasma cells due to vIL-6 overproduction. Detection of vIL-6-expressing cells in the peripheral blood of one patient may represent disseminated infection consistent with a multicentric presentation.

The prevalence of KSHV is higher in Italian populations than in North American or Northern European populations,[22,28,29] and PCR-based studies for KSHV have found 0 to 9% of lymph nodes from Italians with follicular hyperplasia to be positive.[30,31] It is unlikely, however, that our results are due to chance alone. In our study, none of 25 lymph nodes from Italian patients without Castleman's disease were positive for KSHV by PCR or by vIL-6 immunohistochemistry. vIL-6 was present only in KSHV-positive Castleman's disease tissues but in none of 48 KS lesions, indicating that the expression of vIL-6 was restricted to Castleman's disease lesions among KSHV-infected tissues. It is likely that vIL-6 expression is directly responsible for B cell expansion in HIV-negative, KSHV-positive Castleman's lesions, possibly through abrogation of normal B cell apoptotic pathways.[15] While the patients in our study had an aggressive clinical course, studies of HIV-positive patients with KSHV-associated Castleman's disease frequently respond to conventional chemotherapy.[32]

Eight of the Castleman's disease patients in our study had no evidence of KSHV infection. The underlying pathophysiological mechanism for IL-6 overexpression in these patients remains obscure. Two of the eight KSHV-negative patients developed non-Hodgkin's lymphomas, which suggests that endogenous IL-6 overproduction may be a precursor event or may result from the development of non-Hodgkin's lymphoma. Immunological abnormalities including mono-/polyclonal gammopathy, systemic lupus erythematosus, and recurrent infections were also common in the KSHV-negative patients.

vIL-6 is able to substitute for huIL-6 in preventing apoptosis of IL-6-dependent mouse myeloma cells, and its antiapoptotic activity may contribute to the B cell expansion which occurs in Castleman's disease. vIL-6, unlike huIL-6, is able to directly activate intracellular signaling through interactions with the gp130 protein in the absence of IL-6Ra but induces Jak-STAT pathway signaling that is identical to huIL-6.[21] Polyclonal lymphoproliferative disorders caused by the related herpesvirus, EBV, are also characterized by excess huIL-6 production, suggesting that both KSHV and EBV may have developed specific, but distinctive, mechanisms to generate protective IL-6 activity, possibly as a countermeasure against cellular defenses that initiate apoptosis.[15] In addition to Castleman's disease, vIL-6 is also highly expressed in body cavity-based/primary effusion B cell lymphoma but is not significantly expressed in KS lesions.[15]

Castleman's disease infected with KSHV is only a subset of all Castleman's lesions; however, this subset is the first recognized disorder that is likely to be caused by a nonhuman virus-encoded cytokine. The success in using neutralizing monoclonal antibodies against presumed KSHV-uninfected Castleman's disease suggests that specific treatment for vIL-6 overexpression may have palliative effects on KSHV-infected patients. Definitive treatment for the disorder, however, may require therapy to control the underlying viral infection.

Acknowledgments

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