

Short communication

Evidence against KSHV infection in the pathogenesis of multiple myeloma

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is likely to play a pathogenic role in Kaposi's sarcoma, body cavity-based primary effusion lymphoma and a subset of Castleman's disease. A recent polymerase chain reaction (PCR)-based study reported an association between KSHV and multiple myeloma (MM). We searched for KSHV infection in MM patients by serology, PCR and immunohistochemistry. In addition, we cultured dendritic and stromal cells from MM patients. KSHV antibodies were universally absent from MM patients (0/25) whereas EBV antibodies were nearly ubiquitous (24/25). All of the bone marrow biopsies (0/16) and negative controls (0/4) were vIL-6 negative. None of the bone marrow aspirates (0/6) or biopsies (0/3), peripheral blood mononuclear cells (0/8), mononuclear apheresis cells (0/5) or dendritic cell cultures (0/5) were positive by PCR. One of the MM stromal cell cultures (1/7) was positive for KSHV DNA by PCR and weakly positive on direct southern hybridization using a probe to the terminal repeat region. However, this same patient was PCR negative using another primer set, KSHV seronegative, and negative for vIL-6 immunostaining. Our results suggest that the KSHV DNA positivity rate among MM patients is much lower than previously reported. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is a newly discovered herpesvirus (Chang et al., 1994) causally associated with Kaposi's sarcoma (Olsen and Moore, 1997). In addition, KSHV is likely to play a pathogenic role in two other diseases: body cavity-based primary effusion lymphoma (Cesarman et al., 1995) and a subset of Castleman's disease (Soulier et al., 1995). Rettig et al. (1997) reported detection of Kaposi's sarcoma-associated herpesvirus (KSHV) DNA by polymerase chain reaction (PCR) in cultured bone marrow stromal cells of 15 of the 15 multiple myeloma (MM) and two of eight monoclonal gammopathy of undetermined significance (MGUS) patients. In situ hybridization (ISH) for a late lytic KSHV gene product, ORF26 (VP23), in both nuclear and cytoplasmic compartments of most of the cultured stromal cells from MM patients was reported. However, this pattern of gene expression is most consistent with lytic virus replication and is not thought to be compatible with prolonged cell survival. Viral interleukin 6 (vIL-6) RNA transcripts were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) in bone marrow stromal cells from three of the three MM patients. Since cellular IL-6 is known to both stimulate growth and inhibit apoptosis of myeloma cells (Klein et al., 1995; Bataille and Housseau, 1997), Rettig and colleagues speculated that KSHV vIL-6 could play a role in the pathogenesis of MM. The same group has subsequently demonstrated a high rate of KSHV positivity by PCR (six out of seven) and ISH (17 out of 20) using a probe to a latent gene (vcc1) in fresh bone marrow from MM patients (Said et al., 1997). Interestingly, serologic studies find no association between KSHV and MM (MacKenzie et al., 1997; Marcelin et al., 1997; Masood et al., 1997; Parravicini et al., 1997; Whitby et al., 1997).

To investigate discrepancies reported in the literature, we searched for evidence of KSHV infection in patients with MM with a comprehensive battery of assays including serology, immunohistochemistry and PCR. Additionally, we tested a variety of different types of fresh, processed, as

well as cultured tissues obtained by a variety of different methods.

2. Materials and methods

Sera were collected at Columbia Presbyterian Hospital in a blinded fashion from 48 patients with abnormal serum protein electrophoresis levels (SPEP). Fourteen of these patients had MM and nine had MGUS. Eleven additional MM patients were identified and included in the study. These patients were compared to 70 healthy blood donors. Antibodies to latent and lytic KSHV-related antigens were detected by immunofluorescence assay (LANA IFA) and Western blot (ORF65 WB), respectively, as previously described (Gao et al., 1996b; Simpson et al., 1996; Rainbow et al., 1997). Serum were diluted 1:160 for the IFA and 1:100 for the Western blot. The presence of detectable EBNA-1 was used as a surrogate indicator for immunologic response in patient sera. EBV-infected whole P3HR-1 cells were immunoblotted as previously described (Gao et al., 1996a). All sera were coded, tested and read in a blind fashion.

As a conservative assumption (to ensure that weakly reactive sera were not considered negative), faint signals in these assays which we usually interpret as indeterminate or negative were read as positive, but only sera which tested positive with both assays were scored as positive. None of the 25 MM or nine MGUS patients were KSHV seropositive on both assays (Table 1). Patients with abnormally elevated immunoglobulins may have non-specific cross-reactivity and the abnormal SPEP population is likely to contain HIV-positive patients that are independently at-risk for KSHV infection. Three (12%) of the 25 patients with an abnormal SPEP were seropositive on both assays, including one patient with AIDS. One (1%) of the 70 New York City blood donors were seropositive for KSHV on both assays. The results for the individual assays are given in Table 2. There was no significant difference in the KSHV seropositivity rates between MM or MGUS patients and blood donors. The abnormal SPEP population was significantly more likely to

Table 1
Serology, PCR and immunohistochemistry for KSHV

	No. tested	KSHV ^a	EBV ^b
Serology		No. (%) seropositive	
Multiple myeloma	25	0	24 (96)
MGUS	9	0	9 (100)
Abnormal SPEP	25	3 (12) ^d	25 (100)
NYC blood donors ^c	70	1 (1)	66 (94)
PCR DNA		No. (%) KS330 ₂₃₃ positive	
MM bone marrow aspirates	6	0	
MM bone marrow biopsy	1	0	
MGUS bone marrow biopsies	2	0	
Control bone marrow biopsies	1	0	
MM PBMCs	8	0	
MM mononuclear apheresis cells	5	0	
MM dendritic cell cultures	5 ^e	0	
MM stromal cell cultures	7 ^e	1 (14)	
MGUS stromal cell culture	1 ^e	0	
Immunohistochemistry for vIL-6		No. (%) KSHV vIL-6 positive	
MM bone marrow biopsies	16	0	
Normal controls	4	0	
MM stromal cell culture, embedded	1	0	

^a Seropositive by both LANA IFA and ORF65 WB

^b EBNA-1 seropositive by Western blot.

^c Seronegative for HIV 1 and 2, HTLV 1 and 2, hepatitis B and C viruses, and syphilis.

^d Includes one patient with AIDS; HIV status of the other two is unknown.

^e Includes four samples cultured using protocol provided by J. Berenson.

be LANA IFA positive than the blood donors ($P = 0.0005$), however, there was no difference in the ORF65 WB results. All sera tested were positive by WB to the Epstein–Barr virus (EBV) EBNA-1 protein except for one MM patient and

four blood donors indicating that serologic non-responsiveness did not account for our results (Table 1).

3. Results and discussions

Although no standard battery of serologic tests to determine KSHV seropositivity exists, these two well-established assays correlate closely with KSHV infection and are individually greater than 80% sensitive in detecting infection (Gao et al., 1996b; Simpson et al., 1996). Furthermore, our serologic findings are in accord with recent studies conducted in Europe and America (MacKenzie et al., 1997; Marcelin et al., 1997; Masood et al., 1997; Parravicini et al., 1997; Whitby et al., 1997).

Sixteen formalin-fixed, paraffin-embedded bone marrow biopsies from MM patients and four from normal controls were examined for vIL-6 expression by immunohistochemistry using rabbit

Table 2
Serology for KSHV by individual seroassay

Patient population	No. tested	No. (%) seropositive for KSHV	
		LANA IFA	ORF65 WB
Multiple myeloma	25	5 (20)	3 (12)
MGUS	9	2 (22)	1 (11)
Abnormal SPEP	25	11 (44) ^a	5 (20)
NYC blood donors	70	8 (11)	5 (7)

^a Significantly higher seropositivity rate ($P = 0.0005$) when compared to blood donors.

antiserum (vIL-6 R394) raised against two vIL-6 peptides that do not recognize human IL-6 (Moore et al., 1996a). All bone marrow biopsies were negative for vIL-6 antigen expression as seen previously (Moore et al., 1996b). Processing of positive control samples (BCBL-1 cells) by a variety of methods (including decalcification and B5 fixation) had no effect on vIL-6 immunoreactivity as detected by the vIL-6 specific antisera.

Bone marrow aspirates ($n = 6$), peripheral blood mononuclear cells (PBMCs; $n = 8$), mononuclear apheresis cells ($n = 5$), and bone marrow biopsies ($n = 4$), were taken from MM and control patients for PCR-based studies. Bone marrow mononuclear cells and PBMCs were isolated from bone marrow aspirates and blood, respectively, by Ficoll–Hypaque density gradient sedimentation. Mononuclear apheresis cells comprise mostly early hematopoietic cells including CD34⁺ stem cells that exit from the bone marrow to peripheral blood after treatment of patients with cyclophosphamide and granulocyte-colony stimulating factor (G-CSF). The bone marrow biopsies tested were processed fresh in their entirety and immediately put into DNA lysis buffer. DNA was extracted using phenol-chloroform and PCR was performed in a blinded fashion as previously described using KS330₂₃₃ primers (Chang et al., 1994). We used 0.3–1 μ g of DNA per PCR reaction and have previously shown the sensitivity to detect as few as five KSHV copies (Tarte et al., 1998). To ensure the integrity of the DNA, genomic DNA was amplified using human pyruvate dehydrogenase PCR primers (PDH1: 5'-GGG TAT GGA TGA GGA GCT GG-3', PDH2: 5'-TCT TCC ACA GCC CTC GAC TAA-3') or β -globin PCR primers (forward primer 5'-CAACTTCATCCACGTTACC-3', reverse primer 5'-GAAGAGCCAAGGACAGGTAC-3'). PCR on the mononuclear apheresis cells was performed independently in two laboratories.

Similar to findings reported by Rettig et al. and others (Cesarman et al., 1995; Pastore et al., 1995; Masood et al., 1997; Parravicini et al., 1997; Rettig et al., 1997), PCR on fresh bone marrow aspirates and peripheral blood mononuclear cells (PBMCs) from MM patients were negative for viral DNA (Table 1). To address concerns that

stromal cells may not be represented in bone marrow aspirates, we also examined ground up bone marrow biopsies from one MM, two MGUS, and one control patients. In contrast to findings by Said et al. (1997) who found six of the seven MM bone marrows positive for KSHV using KS330₂₃₃, these samples were also negative by PCR.

To address concerns that KSHV positivity of stromal cells can only be detected under enriched culture conditions, we also obtained five dendritic and seven stromal cell cultures from MM bone marrow aspirates and one stromal cell culture from a MGUS patient. The five dendritic cell cultures were generated from patients with MM using adherent apheresis cells after treatment with cyclophosphamide and G-CSF and were cultured for 7 days with IL-4 and GM-CSF in RPMI 1640 and 10% of fetal calf serum. The recovered cells contained approximately 90% dendritic cells (CD1⁺, CD4⁺, CD80⁺, CD68⁺ and CD14⁻) that were able to endocytose and efficiently present soluble antigen to T lymphocytes (Tarte et al., 1997). Dendritic cell cultures from two cancer patients were used as controls. Four stromal cell cultures from patient bone marrows (1 million cells/ml) were prepared in RPMI 1640 (Gibco-BRL) with 10% fetal calf serum (FCS). Four additional stromal cell cultures were prepared using a protocol kindly provided by J. Berenson modified from the methods previously reported (Rettig et al., 1997): Iscove's Modified Dulbecco's Medium (GibcoBRL), 10% fetal bovine serum, 10% horse serum, 1% penicillin-streptomycin, 1% 200 mM L-glutamine, and fungizone. These stromal cells represent a mixed population of 21–38% CD68⁺, CD1a⁻, CD34⁻ and HLA-DR⁺ cells. After 4–5 weeks of culture, confluent adherent cells were trypsinized, DNA was extracted, and PCR was performed as above. PCR on the dendritic cell cultures, as well as three of the stromal cell cultures, was performed independently in two laboratories. PCR using herpesvirus consensus primers was performed on one stromal cell culture as previously described (VanDevanter et al., 1996).

The immunophenotype of the four MM stromal cell cultures grown according to Rettig et al.

(1997) displayed the immunophenotype reported (CD68⁺, CD34⁻, CD1a⁻ and HLA-DR⁺). Only one of these stromal cell cultures was positive on PCR using KS330₂₃₃, however, this sample was negative using KSHV major capsid protein (MCP) primers (Moore et al., 1996b). Direct Southern hybridization using a probe to the terminal repeat region was weakly positive only after 24-h exposure on a phosphoimager. However, this patient was KSHV seronegative, and examination of cultured stromal cells by vIL-6 immunostaining produced non-specific staining. Repeat runs could not confirm the positivity. Preimmune serum was used as a negative control. In addition, PCR using herpesvirus consensus primers was negative. Although this patient may be infected with KSHV, we could not rule out the possibility that the results were due to contamination. Extracted DNA was depleted in our studies and the cell culture was not maintainable precluding further analysis. All other cultures were found to be KSHV negative by PCR.

Direct genomic Southern hybridization for KSHV DNA in MM stromal cell may help to resolve this issue given the level of in situ hybridization detected by Rettig and colleagues. Unlike the ORFK2 (vIL-6) gene, the ORF26 gene encodes a capsid protein which is not transcribed except during late viral gene expression (e.g. lytic virus replication (Miller et al., 1997)). In situ hybridization detection of ORF26 mRNA or DNA in the cytoplasm of all cells of MM stromal cultures by Rettig et al. (1997) implies that these cells are undergoing lytic replication with export of either virion DNA or lytic gene mRNA into the cytoplasm which is unlikely to be compatible with prolonged cell survival. This issue was addressed in a subsequent paper by using a latent gene probe (vcyclin). Nonetheless, we found only one of our MM patients to have possible evidence of KSHV infection, suggesting that the KSHV DNA positivity rate among MM patients is much lower than previously reported. While we could not detect KSHV infection of our MM patients near the rate seen by Rettig et al. (1997), it remains possible that a subpopulation of MM patients may harbor KSHV infection or that a variant of KSHV is present in MM but not detectable by our assays.

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References

- Bataille, R., Harousseau, J.-L., 1997. Multiple myeloma. *New Engl. J. Med.* 336 (23), 1657–1664.
- Cesarman, E., Chang, Y., Moore, P.S., Said, J.W., Knowles, D.M., 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences are present in AIDS-related body cavity based lymphomas. *New Engl. J. Med.* 332, 1186–1191.
- Chang, Y., Cesarman, E., Pessin, M.S., Lee, F., Culpepper, J., Knowles, D.M., Moore, P.S., 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 265, 1865–1869.
- Gao, S.-J., Kingsley, L., Hoover, D.R., Spira, T.J., Rinaldo, C.R., Saah, A., Phair, J., Detels, R., Parry, P., Chang, Y., Moore, P.S., 1996a. Seroconversion of antibodies to Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens prior to onset of Kaposi's sarcoma. *New Engl. J. Med.* 335, 233–241.
- Gao, S.J., Kingsley, L., Li, M., Zheng, W., Parravicini, C., Ziegler, J., Newton, R., Rinaldo, C.R., Saah, A., Phair, J., Detels, R., Chang, Y., Moore, P.S., 1996b. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. *Nat. Med.* 2 (8), 925–928.
- Klein, B., Zhang, X.G., Lu, Z.Y., Bataille, R., 1995. Interleukin-6 in human multiple myeloma. *Blood* 85 (4), 863–872.
- MacKenzie, J., Sheldon, J., Morgan, G., Cook, G., Schulz, T.F., Jarrett, R.F., 1997. HHV-8 and multiple myeloma in the UK. *Lancet* 350, 1144–1145.
- Marcelin, A.-G., Dupin, N., Bouscary, D., Bossi, P., Cacoub, P., Ravaud, P., Calvez, V., 1997. HHV-8 and multiple myeloma in France. *Lancet* 350, 1144.
- Masood, R., Zheng, T., Tulpule, A., Arora, N., Chatlynne, L., Handy, M., Whitman, J.J., Kaplan, M., Dosik, M., Ablashi, D., Gill, P., 1997. Kaposi's sarcoma-associated herpesvirus and multiple myeloma. *Science* 278, 1970–1971.
- Miller, G., Heston, L., Grogan, E., Gradoville, L., Rigsby, M., Sun, R., Shedd, D., Kushnaryov, V.M., Grossberg, S., Chang, Y., 1997. Selective switch between latency and lytic replication of Kaposi's sarcoma herpesvirus and Epstein-Barr virus in dually infected body cavity lymphoma cells. *J. Virol.* 71 (1), 314–324.
- Moore, P.S., Kingsley, L., Holmberg, S.D., Spira, T., Gupta, P., Hoover, D., Parry, J.P., Conley, L.J., Jaffe, H., Chang,

- Y., 1996a. Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. *AIDS* 10, 175–180.
- Moore, P.S., Boshoff, C., Weiss, R.A., Chang, Y., 1996b. Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* 274 (5293), 1739–1744.
- Olsen, S.J., Moore, P.S., 1997. Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) and the etiology of KS. In: Freidman, H., Medveczky, P., Bendinelli, M. (Eds.), *Molecular Immunology of Herpesviruses*. Plenum, New York, pp. 115–147.
- Parravicini, C., Lauri, E., Baldini, L., Neri, A., Poli, F., Sirchia, G., Moroni, M., Galli, M., Corbellino, M., 1997. Kaposi's sarcoma-associated herpesvirus and multiple myeloma. *Science* 278, 1969–1970.
- Pastore, C., Gloghini, A., Volpe, G., Nomdedeu, J., Leonardo, E., Mazza, U., Saglio, G., Carbone, A., Gaidano, G., 1995. Distribution of Kaposi's sarcoma herpesvirus sequences among lymphoid malignancies in Italy and Spain. *Br. J. Haematol.* 91, 918–920.
- Rainbow, L., Platt, G.M., Simpson, G.R., Sarid, R., Gao, S.-J., Stoiber, H., Herrington, S., Moore, P.S., Schulz, T.F., 1997. The 226- to 234-kilodalton latent nuclear protein (LNA) of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) is encoded by ORF73 and is a component of the latency-associated nuclear antigen. *J. Virol.* 71, 5915–5921.
- Rettig, M.B., Ma, H.J., Vescio, R.A., Pöld, M., Schiller, G., Belson, D., Savage, A., Nishikubo, C., Wu, C., Fraser, J., Said, J.W., Berenson, J.R., 1997. Kaposi's sarcoma-associated herpesvirus infection of bone marrow dendritic cells from multiple myeloma patients. *Science* 276, 1851–1854.
- Said, J.W., Rettig, M.R., Heppner, K., Vescio, R.A., Schiller, G., Ma, H.J., Belson, D., Savage, A., Shintaku, I.P., Keoffler, P., Asou, H., Pinkus, G., Schrage, M., Green, E., Berenson, J.R., 1997. Localization of Kaposi's sarcoma-associated herpesvirus in bone marrow biopsy samples from patients with multiple myeloma. *Blood* 90 (11), 4278–4282.
- Simpson, G.R., Schulz, T.F., Whitby, D., Cook, P.M., Boshoff, C., Rainbow, L., Howard, M.R., Gao, S.-J., Bohensky, R.A., Simmonds, P., Lee, C., de Ruiter, A., Hatziakis, A., Tedder, R.S., Weller, I.V.D., Weiss, R.A., Moore, P.S., 1996. Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. *Lancet* 348, 1133–1138.
- Soulier, J., Grollet, L., Oskenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M.-F., Clauvel, J.-P., Raphael, M., Degos, L., Sigaux, F., 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castlemann's disease. *Blood* 86, 1276–1280.
- Tarte, K., Lu, Z.Y., Fiol, G., Legouffe, E., Rossi, J.-F., Klein, B., 1997. Generation of virtually pure and potentially proliferating dendritic cells from non-CD34 apheresis cells from patients with multiple myeloma. *Blood* 90 (9), 3482–3495.
- Tarte, K., Olsen, S.J., Lu, Z.Y., Legouffe, E., Rossi, J.-F., Chang, Y., Klein, B., 1998. Clinical-grade functional dendritic cells from patients with multiple myeloma are not infected with Kaposi's sarcoma-associated herpesvirus. *Blood* 91 (6), 1852–1857.
- VanDevanter, D.R., Warren, P., Bennett, L., Schultz, E.R., Coulter, S., Garber, R.L., Rose, T.M., 1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. *J. Clin. Microbiol.* 34 (7), 1666–1671.
- Whitby, D., Boshoff, C., Luppi, M., Torelli, G., 1997. Kaposi's sarcoma-associated herpesvirus and multiple myeloma. *Science* 278, 1971–1972.