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EXPRESSION OF HHV-8 LATENCY-ASSOCIATED T0.7 RNA IN SPINDLE CELLS AND ENDOTHELIAL CELLS OF AIDS-ASSOCIATED, CLASSICAL AND AFRICAN KAPOSI'S SARCOMA

Michael STÜRZL[1]*, CORNELIA BLASIG[1], Anneliese SCHREIER[1], Frank NEIPEL[2], Christine HOHENADL[3], Emmanuelle CORNALI[1], Gudrun ASCHERL[1], Stefan ESSER[4], Norbert H. BROCKMEYER[4], Marianne EKMAN[5], Ephata E. KAAYA[5], Erwin TSCHACHLER[6] and Peter BIBERFELD[5]

[1] *Abteilung Virusforschung, Max-Planck-Institut für Biochemie, Martinsried, Germany*
[2]*Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Erlangen, Germany*
[3]*Institut für Molekulare Virologie, GSF, Oberschleissheim, Germany*
[4]*Universitätshautklinik, Universität Essen, Essen, Germany*
[5]*Immunopathology Laboratory, Karolinska Institute and Hospital, Stockholm, Sweden*
[6]*Universitätshautklinik, Universität Wien, Vienna, Austria*

Analysis by polymerase chain reaction (PCR) and serological studies have demonstrated a close association between the novel human herpes virus, Kaposi's sarcoma-associated herpes virus (KSHV) or human herpes virus-8 (HHV-8) and the development of Kaposi's sarcoma (KS). To clarify the role of HHV-8 in KS pathogenesis, we investigated at the cellular level by *in situ* hybridization the expression of a recently described 0.7-kb HHV-8-encoded mRNA (T0.7 mRNA) in KS tissues of different epidemiological origin (AIDS-KS, African endemic KS and classical KS). The T0.7 mRNA likely encodes a small membrane protein, supposedly expressed in latently HHV-8-infected cells. Indeed, we detected T0.7 mRNA in virtually all cells of the cell line BCBL-1 established from a body cavity-based lymphoma (BCBL) and latently infected with HHV-8. In all KS biopsies examined, independent of their epidemiological type, the late-stage (nodular) KS tissues showed a high level of T0.7 mRNA expression in typical KS spindle cells but also in endothelial cells lining blood vessels, indicating latent HHV-8 infection of these cells. The presence of T0.7-expressing cells was restricted to KS tumor tissue and therefore appears to indicate an important role of latent HHV-8 infection in KS pathogenesis. *Int. J. Cancer* 72:68–71, 1997.

Kaposi's sarcoma (KS) is usually a multifocally developing tumor with distinct cutaneous and visceral lesions. Histologically, KS is characterized by a prominent vascular component, in a stroma of spindle-shaped cells, considered to represent the tumor cells of the lesions, admixed with infiltrating inflammatory cells (Ensoli *et al.,* 1991; Kaaya *et al.,* 1995; Stürzl *et al.,* 1992*a*). From epidemiological data, it has been suggested that a sexually transmitted infectious agent may be the cause of KS (Beral *et al.,* 1990). A novel human herpes virus (HHV-8) has been isolated from AIDS-associated KS (Chang *et al.,* 1994) and also detected in all other epidemiological forms of KS, both by PCR (Albini *et al.,* 1996; Buonaguro *et al.,* 1996; Chang *et al.,* 1994; Dupin *et al.,* 1995; Huang *et al.,* 1995; Rady *et al.,* 1995; Schalling *et al.,* 1995; Su *et al.,* 1995) and by seroepidemiological studies (Gao *et al.,* 1996; Kedes *et al.,* 1996; Lennette *et al.,* 1996; Simpson *et al.,* 1996). These findings seem to indicate an important role of HHV-8 in KS pathogenesis. Using PCR *in situ* hybridization (PCR-ISH), Boshoff *et al.* (1995) observed that HHV-8 DNA appears to be associated with flat endothelial cells lining vascular spaces as well as with KS spindle cells. It has been reported that HHV-8 genomic DNA in KS lesions is predominantly of a circular structure, which is characteristic for the latent phase of the herpes viral life cycle (Decker *et al.,* 1996). These observations suggest that the majority of KS spindle cells are not productively but latently infected with HHV-8. In order to demonstrate a possible latent HHV-8 infection, *in situ* hybridization was performed with a strand-specific radiolabelled RNA probe for T0.7 mRNA, which encodes for a small (60 amino acids) membrane protein suggested to be expressed during HHV-8 latent infection (Zhong *et al.,* 1996). Our results show that most of the HHV-8-infected KS cells indeed harbored latent virus.

PATIENTS, MATERIAL AND METHODS

Patients

Twelve biopsies of various epidemiological forms of KS were studied (Table I). The AIDS cases (AKS) included 3 homosexual male patients from Europe and one from Africa. All these patients were of group C according to the Center for Disease Control (CDC) classification (Center for Disease Control, 1992). From 2 of the European AKS patients, 2 biopsies in different stages of development (patch and nodular stage) were examined. Four different biopsies from one patient with classical KS (CKS) were also studied. African endemic KS (EKS) biopsies were obtained from 2 male patients proven to not be infected with HIV. All biopsies were removed for diagnostic purposes with informed consent by the patients. None of the patients was receiving anti-KS therapy.

One control biopsy was obtained from an uninvolved area of the skin of one of the European AKS patients.

Cell culture

The cell line BCBL-1 was originally established from a body cavity-based lymphoma (Komanduri *et al.,* 1996). These cells are latently infected with HHV-8, while no Epstein-Barr virus (EBV) DNA has been found in this cell line (Renne *et al.,* 1996). The BCBL-1 cell line was kindly provided by Dr. Ganem (University of California, San Francisco, CA) and was cultured in RPMI-1640 supplemented with 10% FCS, 0.05 mM 2-mercaptoethanol, 0.02% $NaHCO_3$ and 50 µg/ml penicillin at 37°C in 5% CO_2. For *in situ* hybridization, the cells were centrifuged and washed twice in PBS, resuspended in PBS (1×10^6 cells/ml), dropped onto a silan-coated slide, air-dried and fixed in 4% buffered paraformaldehyde.

In situ *hybridization*

In situ hybridization was carried out as described elsewhere using [^{35}S]-labelled *in vitro*-transcribed RNA complementary to T0.7 mRNA as a probe (Stürzl *et al.,* 1995, 1992*b*). As a control, radiolabelled T0.7 sense strand RNA was used.

The T0.7 cDNA was amplified by PCR using primers binding to the 5′ and 3′ ends of the published T0.7 sequence (Zhong *et al.,* 1996) and inserted into the transcription plasmid pBluescript SK^- (Stratagene, La Jolla, CA).

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*Correspondence to: Abteilung Virusforschung, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, 82152 Martinsried, Germany. Fax: +49-89-8578-3777. E-mail: STUERZL@Biochem.mpg.de

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TABLE I – DETECTION OF T0.7 EXPRESSION IN DIFFERENT EPIDEMIOLOGICAL FORMS OF KS[1]

KS biopsies	Specimen numbers	HIV (+/−)	HHV-8 T0.7 (+/−)
AIDS-associated	**6**		
European KS			
Patch KS stage	3	+	3/3 (+/−)
Nodular KS stage	2	+	2/2 (+)
African KS			
Nodular KS stage	1	+	1/1 (+)
Non-AIDS-associated	**6**		
Classical KS			
Nodular KS stage	4	−	4/4 (+)
African endemic KS			
Nodular KS stage	2	−	2/2 (+)

[1]Specimen numbers: total number of specimens studied in the various groups of patients. HIV (+/−) indicates whether respective patients were HIV-infected (+) or not (−). HHV-8 T0.7 (+/−) gives the numbers of biopsies where T0.7 mRNA was detected in comparison with the number of total biopsies studied. (+), majority of cells were positive; (+/−), only a few cells were positive.

RESULTS

Following strand-specific *in situ* hybridization, a strong hybridization signal was seen in autoradiographs of most BCBL-1 cells, indicating that the corresponding gene is expressed in virtually all of these cells latently infected with HHV-8 (Fig. 1*a*, bright-field, arrows; Fig. 1*b*, dark-field, arrows). Expression levels varied from very high (Fig. 1*a*,*b*, lower arrows) to moderate (Fig. 1*a*,*b*, upper arrows), which may be due either to the fact that BCBL-1 cells used were not synchronized, or to loss of mRNA from some cells during the fixation procedure. No specific labelling was observed in control hybridizations of BCBL-1 cells using the sense strand RNA probe (data not shown), which demonstrated that T0.7 mRNA was specifically detected, but not viral DNA. Overall, the results showed that the T0.7 transcript is a suitable marker for *in situ* detection of cells latently infected with HHV-8. We therefore examined KS biopsy sections of European and African AKS, African EKS and classical KS. Clearly, specific labelling was obtained with the T0.7 antisense probe in approximately 50–70% of tumor cells of all nodular KS lesions examined (Fig. 2*a*, bright-field exposure; 2 positive cells are marked with an arrow; Fig. 2*b*, corresponding dark-field exposure). The T0.7 mRNA-expressing cells appeared to be predominantly of a spindle-cell type (Fig. 2*c*, arrow) and as endothelial-like cells lining non-neoplastic framework vessels in the tumor areas (Fig. 2*d*, arrow). Moreover, T0.7-expressing spindle cells were more frequently seen in well-vascularized areas (Fig. 2*e*, arrow), compared to tumor areas with prominent bundles of spindle cells but with an inconspicuous vasculature (Fig. 2*e*, asterisk). In contrast to the high numbers of cells expressing the T0.7 transcript in nodular KS lesions, relatively few cells (approximately 1–3% of all tumor cells) showed a positive reaction in early-stage patch KS lesions (Fig. 2*g*, bright-field, arrow; Fig. 2*h*, corresponding dark-field exposure). The expression of T0.7 mRNA was practically restricted to the KS tumor area (Fig. 2*f*, right). In peri-lesional tissue of all cases examined, no signal for T0.7 mRNA was observed, neither in the epidermal layer overlaying KS (Fig. 2*i*), nor in fibrocytic cells of the dermis (Fig. 2*i*, left) or in cells associated with blood vessels (Fig. 2*j*, arrow). Similarly, no specific labelling was obtained with the T0.7 antisense probe applied to sections of a KS-free skin region of an AKS patient (Fig. 2*k*). Finally, KS sections consecutive to that shown in Figure 2*a*,*b* did not show any specific signal when hybridized with the T0.7 sense strand RNA probe (Fig. 2*l*).

In summary, HHV-8 T0.7 mRNA was specifically expressed in biopsies from different epidemiological forms of KS (6 AIDS-associated KS, 2 African endemic KS and 4 classical KS) at various stages of development (Table I). The T0.7 transcript was detected in all KS lesions examined, but the relative numbers of T0.7-expressing cells were clearly higher in advanced nodular stages than in early patch stages.

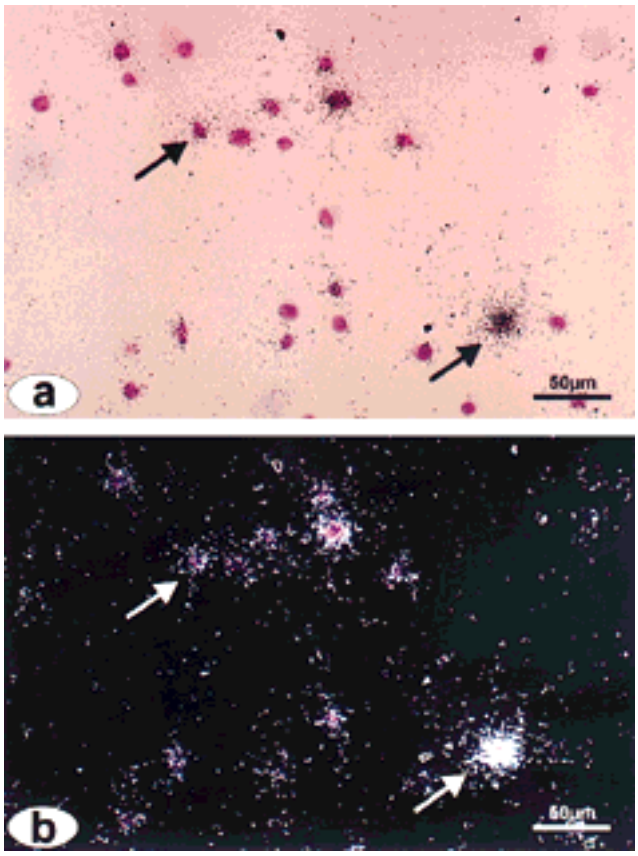

FIGURE 1 – The majority of latently HHV-8-infected BCBL-1 cells express T0.7 mRNA. Strong expression of T0.7 mRNA can be observed in BCBL-1 cells latently with HHV-8 by *in situ* hybridization using strand-specific radiolabelled RNA probes (sp. act. 1×10^9 cpm/µg DNA). (*a*) Bright-field exposure. (*b*) Dark-field exposure. Positive cells are indicated by arrows.

DISCUSSION

In situ hybridization (ISH) experiments clearly indicate that HHV-8 is indeed present in KS spindle cells and vessel-associated cells of KS lesions. This is in agreement with earlier data on the cellular association of HHV-8 in KS biopsies obtained by PCR-ISH (Boshoff *et al.*, 1995). Since the DNA of the tissues examined in our study was not denatured prior to hybridization and no signals were obtained with the sense RNA strand, we conclude that T0.7 mRNA, rather than viral DNA, was detected.

Decker *et al.* (1996) reported that HHV-8 genomic DNA is present in KS lesions predominantly in a circular form, which indicates that most of the cells harboring HHV-8 in KS lesions may be latently infected. Here we show that T0.7 mRNA is synthesized in BCBL-1 cells latently infected with HHV-8, and that this transcript is also present in numerous spindle cells and endothelial-like cells of KS lesions. Altogether, these data indicate that T0.7 mRNA is a latency-associated transcript and that the KS spindle cells and endothelial cells of KS lesions are latently infected by HHV-8.

During the preparation of this manuscript, similar findings on T0.7 expression in AIDS-associated KS lesions were reported by Staskus *et al.* (1997). Our results additionally demonstrate that these observations apply to all different epidemiological forms of KS, including European and African AIDS-associated KS as well as African endemic and classical KS, *i.e.*, that in all KS forms latent HHV-8 infection predominates, as indicated by the expression of T0.7 mRNA.

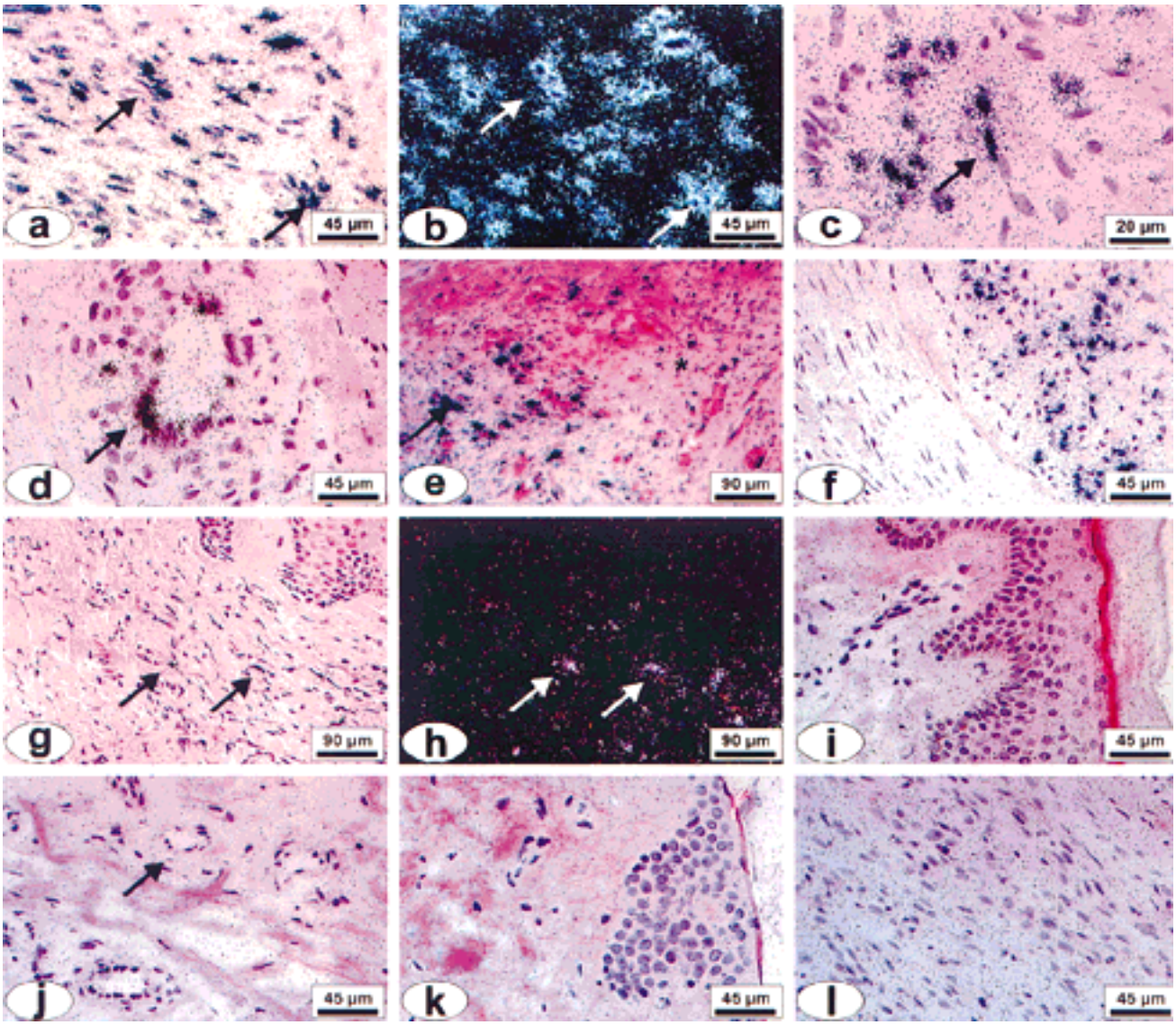

FIGURE 2 – Synthesis of T0.7 mRNA in KS lesions and control tissue. Hybridization with a radiolabelled T0.7 antisense RNA probe. Robust signals were observed in nodular KS lesions. (*a*) Bright-field exposure. (*b*) Dark-field exposure. (*c*,*d*) Higher magnification of T0.7-positive spindle cells (*c*, arrow) and endothelial cells surrounding non-neoplastic blood vessels (*d*, arrow). Strong signals are especially observed in the spindle cells of highly vascularized areas of nodular KS lesions (*e*, arrow), whereas areas with inconspicuous vasculature but with prominent bundles of spindle cells exhibit only a few positive cells (*e*, asterisk). Expression of T0.7 mRNA is restricted to the KS tumor area (*f*, right). Only a few T0.7-positive cells were observed in early KS lesions (*g*, arrow, bright-field). Signals for T0.7 transcripts in a few positive cells are clearly seen in the corresponding dark-field exposure areas (*h*, arrow, dark-field). The epidermal layer overlying the KS (*i*, right), fibrocytic cells of the dermis (*i*, left) and blood vessels outside of the KS lesion (*j*, arrow) did not show any signal. No signals were observed in a biopsy of healthy skin area from a KS patient (*k*). Hybridization with a radiolabelled T0.7 sense RNA probe: hybridization of T0.7 sense RNA to a KS section consecutive to the one shown in (*a*) did not reveal any signal (*l*).

In addition, we noticed a striking difference in the relative numbers of HHV-8-infected cells in early (1–3% of tumor cells positive) compared to advanced (50–70% of tumor cells positive) stages of KS lesions. This supports our interpretation of spindle cells as the predominant target of latent HHV-8 infection, since these cells are rare in early stages and accumulate during the development of KS lesions to the nodular tumor stage (Kaaya *et al.*, 1995; Stürzl *et al.*, 1992*a*). Actually, the survival and proliferation of KS spindle cells may depend on latent infection by HHV-8. Our results also indicate that the presence of HHV-8 in the skin is restricted to the KS tissue. This indicates that HHV-8 is an important pathogenic factor in the development of KS. However, it is not clear whether this agent triggers the local development of KS or is recruited secondarily into the lesions and thereby promotes progression to late tumor stages. Our finding of the T0.7 transcript in only a few cells of early patch lesions favors the second hypothesis. However, it is possible that T0.7 mRNA synthesis in cells of early KS lesions latently infected with HHV-8 is lower than in cells of progressed lesions. Therefore, differences in the relative numbers of HHV-8-positive cells in the different stages of KS evolution may be related to the sensitivity of *in situ* hybridization.

HHV-8 codes for several genes that exhibit interesting biological activities (cyclin D, bcl-2, IL-6, IL-8R, MIP-1α, MIP-1β; F. Neipel, personal communication). The possible expression of these

genes in latently infected KS spindle cells or endothelial cells could thus be of importance for the regulation of KS progression.

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