Presence of Chromosomal Abnormalities and Lack of AIDS Retrovirus DNA Sequences in AIDS-associated Kaposi’s Sarcoma

Pasquale Delli Bovi, Emilio Donni, Daniel M. Knowles II, Alvin Friedman-Kien, Paul A. Luciw, Dino Dina, Riccardo Dalla-Favera, and Claudio Basilisco


ABSTRACT

The frequent occurrence of Kaposi’s sarcoma (KS) in association with the acquired immune deficiency syndrome (AIDS) could be due to the fact that the etiological agent of this tumor is the same retrovirus causing AIDS, to another oncogenic virus frequently found in AIDS patients, or to the unmasking of the tumorigenic potential of KS cells by immunosuppression. We have therefore investigated the presence of DNA sequences homologous to the AIDS retrovirus, cytomegalovirus (CMV), and hepatitis B virus in 13 KS necropsies and biopsies from AIDS patients. All KS DNA samples were negative for AIDS retrovirus or hepatitis B DNA sequences. Two DNAs from necropsies contained CMV DNA, but the data suggested the presence of replicating CMV DNA due to generalized infection. We have also studied cell cultures derived from KS skin biopsies of AIDS patients. These cultures had a short lifetime in vitro and expressed some markers of endothelial cells. The cells were not tumorigenic in nude mice but contained a number of chromosomal rearrangements which were often monochonal within the same culture. However, these abnormalities were different from culture to culture and even in cultures from the same biopsy. The presence of these chromosomal abnormalities seemed to correlate with the cell positivity for endothelial markers. Taken together these results indicate that neither the AIDS retrovirus, CMV, or hepatitis B virus is directly responsible for the altered growth of KS cells, that KS may be polyclonal even within the same lesion, and that KS cells have a tendency to karyotypic rearrangements.

INTRODUCTION

The recently identified epidemic of AIDS is associated with a significant increase in the occurrence of some tumors, namely KS and B-cell lymphomas (1–4). Thus, AIDS and its associated neoplasms may represent a unique model system to study the immunological, virological, and genetic factors which influence the development of malignancies. KS is a multifocal neoplasm, generally considered an angiosarcoma of endothelial origin (5), which presents itself as s.c. nodules or pigmented plaques of the skin or oral mucosa. This tumor often has a rather benign clinical course, although an aggressive tumor often has a rather benign clinical course, although an aggressive physical course, and that KS cells have a tendency to karyotypic rearrangements.

MATERIALS AND METHODS

Tumor Biopsy Processing and Cell Culture Conditions. Cell cultures were derived from KS biopsies using the technique described by Folkman et al. (8), for the isolation of skin capillary endothelial cells with slight modifications. After the specimens were processed and treated with collagenase as described (8), the pellet of cells obtained was resuspended in E199 medium containing 10% fetal calf serum, distributed in two 60-mm Falcon Primaria dishes, and incubated at 37°C. One to 2 h later the medium and unattached cells were removed and replaced with Medium E199 containing 20% fetal calf serum, heparin (Sigma, St. Louis, MO) (90 µg/ml), and endothelial cell growth supplement (Collaborative Research, Lexington, MA) (60 µg/ml) (9). Medium was replaced every 2 days. Microcolonies of nonfibroblast-looking cells were identified, removed, and replated in individual Falcon Primaria dishes or normal tissue culture dishes coated with a 1.5% gelatin solution in Dulbecco’s phosphate-buffered saline. The same medium was used for all further passages.

Immunofluorescence Staining. The cultures were tested for the presence of specific cell markers by indirect immunofluorescent staining of cells fixed on glass cover slips. For Factor VIII-related antigen, Ulex europaeus Agglutinin I, and HLA-DR, fixation was in acetone for 20 min at —20°C. For vimentin and keratin, the fixation was performed in acetone:methanol (3:1) for 20 min at 20°C. For OKM5, fixation was in acetone for 20 min at 20°C. The Factor VIII-related antigen antibody was from Calbiochem (San Diego, CA); U. europaeus Agglutinin I was from Vector Laboratories (Burlingame, CA); the mouse monoclonal antibodies to human HLA-DR and OKM5 were from Dr. C. Y. Wang (Sloan Kettering Institute, New York, NY) and from Ortho Pharmaceuticals, Inc. (Raritan, NJ), respectively; the antibodies against vimentin and keratins were kindly provided by Dr. R. L. Lynam (Department of Pharmacology, N.Y.U. Medical Center) and T. T. Sun (Department of Dermatology, N.Y.U. Medical Center), respectively. Goat anti-rabbit and sheep anti-mouse fluorescein isothiocyanate-conjugated γ-globulins were from Cappel Laboratories (West Chester, PA).

Cytogenetic Analysis. As soon as a sufficient number of dividing cells was available, chromosome preparations were obtained after treatment of cell cultures with colchicine (0.04 µg/ml) for 2 to 3 h. Whenever possible, a minimum of 20 metaphases was counted for each culture, and 5 to 10 karyotypes were prepared from well-spread GTG-banded metaphases (10).

DNA Preparation and Filter Hybridization. The DNAs from both necropsies and biopsies were extracted in the following manner: the specimens were cleaned of all extraneous nontumorous tissues; washed in buffered saline; and then minced with a scalpel. The tissue was resuspended in lysis buffer [10 mM Tris (pH 7.9):10 mM NaCl:10 mM EDTA:0.5% sodium dodecyl sulfate:proteinase K (500 µg/ml)]. The lyse was incubated overnight at 50°C. The DNAs were extracted twice with phenol, once with phenol:chloroform:isoamyl alcohol, and once with chloroform:isoamyl alcohol. Following addition of sodium acetate to a final concentration of 300 mM, the DNAs were alcohol precipitated and washed several times in 75% alcohol. The isolated DNAs were digested with the appropriate restriction enzyme, size fractionated by agarose gel electrophoresis, and transferred to nitrocellulose paper.

Received 4/28/86; revised 8/21/86; accepted 9/3/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This investigation was supported by USPHS Grant CA37295 from the National Cancer Institute.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: AIDS, acquired immune deficiency syndrome; KS, Kaposi’s sarcoma; ARV, acquired immune deficiency syndrome retrovirus; cDNA, complementary DNA; CMV, cytomegalovirus; HBV, hepatitis B virus; HIV, human immunoendoral cord vein endothelia.

6333
For detecting AIDS-related retrovirus sequences, the filter was hybridized to an entire ARV-2 provirus probe (obtained by gel purification of the 9.5-kilobase EcoR I proviral insert from the recombinant plasmid, pARV-λ 7a/2, representing the entire ARV-2 genome circularly permuted at the EcoR I site (11)) and nick translated to a specific activity of $3 \times 10^{8}$ cpm/μg of DNA. Hybridization conditions and washing were as described (11). The filter was then exposed to Kodak XAR-2 film in the presence of an intensifying screen at -70°C. The same filters were generally rehybridized to a human N-ras cDNA probe (12). The entire pN-ras plasmid was nick translated to a specific activity of 2 x $10^{8}$ cpm/μg of DNA. For detecting CMV sequences, the filters were hybridized to two different human CMV (strain Towne) DNA probes cloned in PBR322 plasmid, precisely pRL103 and pRL3. The recombinant plasmid, pRL103, contains a 21.5-kilobase HindIII insert [corresponding to the “C” fragment of the physical map for HindIII (13) cleavage of human CMV strain Towne], and pRL3 contains a 16-kilobase BamHI insert [corresponding to the “C” fragment of the physical map for BamHI cleavage of human CMV strain Towne]. They were kindly provided by Dr. G. S. Hayward. The two fragments represent different parts of the unique large sequences of the human CMV strain Towne. The 21.5-kilobase HindIII fragment represents an immediate early gene and has focus formation activity (Refs. 24 and 25; Footnote 4). The 16-kilobase BamHI fragment represents late sequences (13, 14). The probes were labeled with 32P by nick translation at a specific activity of $3.0 \times 10^{8}$ cpm/μg. The hybridization and washing conditions were those described by Wahl et al. (15).

RESULTS AND DISCUSSION

Presence of Viral Sequences in KS DNA. The frequent occurrence of KS in AIDS patients could be due to the fact that the etiological agent of KS is the same retrovirus causing AIDS (designated LAV, HTLV III, or ARV in different laboratories) (11, 16-19), to another infectious agent with oncogenic potential frequently found in AIDS patients or, in general, in immunosuppressed individuals, or could be merely due to the immunosuppression which unmasksthe unregulated growth properties of cells having genetic alterations that are normally kept in check by the host immune surveillance mechanisms.

We have therefore investigated the presence of ARV DNA (11) in total DNA extracted from KS biopsies and necropsies, as well as in cell cultures derived from KS biopsies (see later). The results of Southern blots with an ARV DNA probe are shown in Fig. 1. All cellular DNAs tested (a total of 13 biopsies and necropsies and 2 cell lines) were uniformly negative for ARV DNA sequences at levels of sensitivity that should have detected approximately 0.2 ARV genome equivalents/cell. To ensure that the various DNAs had been retained by the filters and undergone proper enzyme digestion, the blots were also hybridized to a human N-ras cDNA probe (12). The expected human DNA restriction fragments hybridizing to the N-ras probe were detected in all samples. It appears therefore that the AIDS retrovirus is unlikely to be directly involved in the etiology of AIDS-associated KS.

We next turned our attention to human CMV and hepatitis B virus, since both of these viruses have been implicated in KS (20-23). All KS DNA samples were negative for HBV DNA sequences (data not shown), but two DNA samples from KS necropsies contained sequences which hybridized to CMV DNA probes. Fig. 2 shows the results obtained with one sample. CMV DNA sequences are present in relatively high abundance (approximately 5 copies per cell genome equivalent), and all expected viral DNA fragments homologous to the probes used appeared to be present (13, 14), including those hybridizing to another probe (not shown) corresponding to the CMV repeated sequences. The other DNA sample produced similar results, but the CMV DNA sequences appeared to be present in lower amounts (approximately 2 copies/cell, data not shown). Only 2 of the 13 cases tested contained CMV DNA sequences. In addition, the DNA preparation containing the highest amounts of CMV sequences was derived from the skin necropsy of a patient who had died of CMV infection. These findings therefore are likely to reflect the occurrence of opportunistic CMV infections which are frequently observed in AIDS patients and to be due to the presence of replicating CMV DNA in some cells of the original biopsy, rather than of specific CMV-transforming genes (24, 25) in all tumor cells.

Taken together these results suggest that neither the AIDS sequences. The other DNA sample produced similar results, but the CMV DNA sequences appeared to be present in lower amounts (approximately 2 copies/cell, data not shown). Only 2 of the 13 cases tested contained CMV DNA sequences. In addition, the DNA preparation containing the highest amounts of CMV sequences was derived from the skin necropsy of a patient who had died of CMV infection. These findings therefore are likely to reflect the occurrence of opportunistic CMV infections which are frequently observed in AIDS patients and to be due to the presence of replicating CMV DNA in some cells of the original biopsy, rather than of specific CMV-transforming genes (24, 25) in all tumor cells.

Taken together these results suggest that neither the AIDS
alterations of KS cells would be facilitated by the availability of cell lines originating from these tumors. Experiments carried out with DNA from biopsies and necropsies, although useful, suffer from limitations due to the fact that the proportion of tumor cells in the samples may be low, the supply of DNA is limited, and the properties of the tumor cells themselves cannot be easily studied. For these reasons, we have attempted to obtain cell cultures derived from KS tumors. All patients from whom these cultures were derived had not yet undergone any treatment. The techniques used to establish KS cell cultures were similar to those used for culturing endothelial cells and are described in “Materials and Methods.” The procedure involved the isolation of microcolonies of cells that were morphologically distinct from the background of rapidly growing fibroblasts. Thus, our KS cultures are generally “clonal” in nature, or they originate from a small number of cells.

Cells cultured from KS biopsies were propagated in the medium described, and their properties were studied in a variety of ways to attempt to determine their relationship to the primary tumor cells and to assess their degree of transformation in vitro and in vivo. The cultures were compared to human skin fibroblasts, normal HUVE, and SV40-transformed HUVE cell cultures. The growth rate of cells in cultures was variable. Division time was on the order of 24 to 30 h; most cultures slowed their growth rate after six or seven passages and stopped growing after about 2 mo. One culture (L-9) has been maintained for over 4 mo (approximately 70 cell generations). DNA extracted from L-9 cells did not contain any sequences homologous to ARV or CMV DNA probes (see above). The presence of specific cell markers was tested by immunofluorescence or immunoperoxidase reactions on fixed cells (Table 1). All cells were negative for keratins but positive for vimentin, indicating their mesenchymal origin (26). The presence of Factor VIII-related antigen, a marker of endothelial cells (27, 28), was not detected in any of the cultures. U. europaeus type I agglutinin (29, 30) did not react with most cultures, but reacted with a few cells in Bart 3 that, however, were no longer detectable at later passages.

The lack of Factor VIII positivity in the cells was consistent with the observation that Factor VIII is not uniformly present in KS sections (30–32), particularly in the so-called spindle cells; also SV40-transformed capillary (33) and HUVE cells (Ref. 34; Footnote 5) become Factor VIII negative after a few passages in culture. The KS cell cultures were assessed for the presence of HLA-DR antigens (35, 36) and the antigen(s) recognized by OKMS, a monoclonal antibody which has been shown to stain specifically the surface of capillary endothelial cells (27, 28), was not detected in any of the cultures. U. europaeus type I agglutinin (29, 30) did not react with most cultures, but reacted with a few cells in Bart 3 that, however, were no longer detectable at later passages.

The positivity for OKMs-5 and HLA-DR markers by endothelial cells as well as KS indicates that the cultures described here are likely to be

---

Footnote 5: Personal observation.
derived from KS spindle cells. It is interesting to note that, while normal endothelia are positive for both these markers, our KS cultures tested so far appeared to react with OKM5 or anti-HLA-DR antibodies, but not with both.

Karyotypic analysis of the KS cell cultures revealed variable chromosomal aberrations in different cultures (Fig. 3; Table 1). Metaphases from the L-9 cell cultures showed a partial trisomy 17 due to a selective duplication of the segment 17q11→qter. This extra material was translocated to the short arm of chromosome 14, resulting in a 46,XY,-14,+der(14)t(14;17)(p13;q11) karyotype. This karyotype was observed in all metaphases examined. The KS 19-1 cultures had a t(19;21)(q13;q11) translocation, while the KS 19-2 and -3 cultures had several numerical and/or structural rearrangements, but not the t(19;21) translocation. The 6q-,8p+,13q- anomalies were present in both these latter cultures, suggesting a clonal evolution. Analysis of the KS B-3 culture revealed three cytogenetic clones. The first (approximately 60% of the metaphases) had a normal 46,XY karyotype, the second (30%), a normal diploid chromosome number with a 12p+ chromosome derived from an end-to-end duplication of the segment 12p11→p13, while the third (about 10% of the cells) showed a near tetraploid karyotype in which the only common anomaly was a t(12;12) translocation.

The karyotypic abnormalities of our KS cultures contrasted with the situation in HUVE cells or skin fibroblast cultures, which showed no detectable chromosomal aberrations. The same was true of those cultures derived from KS samples (e.g., L-4 and KS26-1) that had been considered to be mainly composed of normal fibroblasts on the basis of morphology and failure to react with either OKM5 or anti-HLA-DR antibodies. These findings suggested that KS cells are not characterized by specific chromosomal abnormalities. However, the presence of a different chromosomal rearrangement in all KS cultures suggests a tendency towards chromosomal rearrangements. To determine whether this situation reflected the development and clonal evolution of cells carrying chromosomal abnormalities during propagation in culture, or whether those cells existed in the tumor itself, we examined two KS primary cultures. In the case of the KS26 mass culture, we found a 8q deletion in one out of 16 metaphases examined. In KSB-mess culture we found several chromosomal abnormalities, but unfortunately the poor quality of banding did not allow a satisfactory interpretation of the karyotypes. Two later analyses of both these mass cultures did not reveal any clonal evolution of the cells with abnormal karyotypes. We could never detect any metaphase in fresh tumor cells.

The presence of chromosomal abnormalities in the KS cell cultures described here suggests that these cells may have some of the properties of tumor cells (39). However, the morphology of these cultured cells and their growth patterns did not appear transformed, and the cultures (with the possible exception of L-9) did not have an indefinite growth potential but underwent senescence rather rapidly. The cells did not exhibit any of the properties commonly associated with transformation in vitro, such as growth in soft agar medium, and were not tumorigenic in nude mice (at 10⁶ cells/mouse) (not shown). The lack of transformed growth properties may be due to suboptimal growth conditions. A variety of growth factors and hormones are being tested to determine whether any of them would influence growth properties in these cultures.

Table 1 Histochemical and karyotypic characteristics of cell cultures derived from KS

<table>
<thead>
<tr>
<th>Cells</th>
<th>Vimentin</th>
<th>Keratins</th>
<th>Factor VIII*-related antigen</th>
<th>U. europaeus* Agglutinin I</th>
<th>HLA-DR* (la)</th>
<th>OKMS*</th>
<th>Frequency of abnormal karyotyped metaphases</th>
<th>Chromosome analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVE XIII†</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>0/7 (30) 46,XY</td>
<td></td>
</tr>
<tr>
<td>HUVE-SV‡</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>(15) Major karyotypic abnormalities</td>
<td></td>
</tr>
<tr>
<td>FS4†</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/6 (40) 46,XY</td>
<td></td>
</tr>
<tr>
<td>KS L†</td>
<td>L-4</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>0/5 (15) 46,XY</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L-9</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>10/10 (40) 46,XY/-14, +der(14)(t(14;17)(p13q11)</td>
<td></td>
</tr>
<tr>
<td>KS 19‡</td>
<td>19-1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>10/10 (19) 46,XY(t(19;21)(q13;q11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19-2</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>3/3 (21) 46,XY, 6q-, 8p+, inv(10)(q22.1q24.3), 12p+, 13q-, 14q+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19-3</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5/5 (22) 49,XY, +2, +7, +10q-, +8p, +13q-</td>
<td></td>
</tr>
<tr>
<td>KS 26‡</td>
<td>26-MC</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>1/11 (16) 46,XY, 46,XY, del(8)(q21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26-1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/7 (25) 46,XY</td>
<td></td>
</tr>
<tr>
<td>KS B‡</td>
<td>B-1</td>
<td>NT*</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>2/5 (18) 46,XY/46,XY, 8p-, +46,XY, +17, -19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-2</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>0/4 (23) 46,XY</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B-3</td>
<td>NT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7/9 (46) 46,XY(60%)/46,XY, 12p+ (30%) /</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>91, XXXY→12, +17, +der(12), (12;12)(p13p13)(10%)</td>
<td></td>
</tr>
</tbody>
</table>

* Specific cell markers used in the characterization of the cell cultures.
† The karyotype of most KS cultures was determined between the second and third passage in vitro. For HUVE cells, HUVE-SV, and FS4, the karyotype was determined at the 13th passage in vitro or after the cells had been in culture for approximately 3 mo, respectively.
‡ Normal human umbilical cord vein cells at the 13th passage in vitro.
§ Human umbilical cord vein cells transformed with a plasmid containing the SV40 early region.
* Human foreskin fibroblasts.
* Individual biopsies from KS lesions (all from different patients) from which each culture indicated below was originated.
* NT, not tested.
Conclusions

The absence of DNA sequences homologous to those of the AIDS-associated retrovirus or of hepatitis B virus in all of the KS samples we tested argues against a direct viral etiology for KS. Although 2 samples of 13 contained CMV DNA, the fact that the whole CMV genome was present and the number of copies was relatively high suggested an opportunistic infection, rather than a direct causative role of this virus in KS. We cannot rule out the presence of minute fragments of ARV, HBV, or CMV DNA integrated into the DNA of KS cells, nor the possibility that these viruses may have acted by a “hit and run” (40, 41). Our results, however, suggest that immunosuppression may be the main factor determining the frequent occurrence of KS in AIDS patients. Subtle genetic alterations in KS cells or abnormal concentrations of growth factors may lead to proliferation and dedifferentiation of skin endothelial cells, a phenomenon that normally would be kept in check by the immune system. Accordingly, the phenotype of the cells in the KS cultures is not typically “transformed,” and these cells exhibit only some markers characteristic of endothelial cells. Most importantly, they appear to have undergone chromosomal rearrangements which were not “specific” and varied from culture to culture, including cultures derived from a single biopsy (e.g., KS19-1 and -2).

We could not assess whether the chromosomal abnormalities discussed above were already present in the original KS lesions or whether they arose during propagation in culture. We found, however, several cultures (most notably L-9 and KS19-1) in which all cells contained the same characteristic chromosomal alteration, suggesting a tendency toward chromosomal rearrangement and clonal evolution in these cells. Since the small
colonies which originated them were picked a few days after plating the tumor in culture, these rearrangements probably existed in the original cell population. In other cases, however, not all the cells in the culture carried detectable chromosomal rearrangements. This indicates a considerable heterogeneity in the cell populations making up the KS lesion; chromosomal rearrangements could occur at different stages of growth, both in the tumor and in tissue culture. Taken together these observations suggest that KS is a multifocal tumor that is polyclonal even within the same skin lesion.

The presence of many different chromosomal aberrations in our KS cell cultures could be also be due to a phenomenon of generalized chromosomal instability in our patients. In this respect, it is interesting to note that recently nonspecific chromosomal aberrations have been reported in lymphocytes from patients with sporadic KS, as well as in "fibroblast-like" cells derived from KS lesions of the same patients (42). This could suggest that KS is associated with a yet unidentified virus which could cause chromosomal instability.

ACKNOWLEDGMENTS

We thank Dr. G. Teebor and Dr. M. Ittmann for providing us with some KS specimens and Dr. D. Moscatelli for the helpful suggestions and technical advice on endothelial cell cultures.

REFERENCES


Presence of Chromosomal Abnormalities and Lack of AIDS Retrovirus DNA Sequences in AIDS-associated Kaposi's Sarcoma

Pasquale Delli Bovi, Emilio Donti, Daniel M. Knowles II, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/12_Part_1/6333

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.