monoclonal antibody having a different specificity. A logical step in the development of a clinically useful antitumor agent would be to insert the hypervariable sequences from antitumor antibodies generated in mice into a single-chain antigen-binding protein derived from human framework regions.

Single-chain antigen-binding proteins are expected to have advantages in clinical applications because of their small size. These proteins should be cleared from serum faster than monoclonal antibodies or Fab fragments. Because they lack the Fc portion of an antibody, which is recognized by cell receptors, they should have a lower background in imaging applications and they should be less immunogenic. They may penetrate the microcirculation surrounding solid tumors better than monoclonal antibodies. We foresee the use of single-chain antigen-binding proteins in applications for which monoclonal antibodies and antibody fragments are currently used, such as (i) imaging and therapy of cancers and cardiovascular or other diseases, (ii) separations, and (iii) biosensors.

The peptide linkers present in single-chain antigen-binding proteins can be designed with specialized function such as sites for the chelation of metals or the attachment of drugs or toxins for applications in imaging and therapy. In addition, it will be possible to design sequences into the linkers or at the carboxyl terminus for the attachment of the protein to solid supports for use in clinical assays, separations, and sensing devices.

Note added in proof: Since this report was submitted another paper dealing with similar technology has appeared (*17*).

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Kaposi's Sarcoma Cells: Long-Term Culture with Growth Factor from Retrovirus-Infected CD4⁺ T Cells

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Studies of the biology and pathogenesis of Kaposi's sarcoma (KS) have been hampered by the inability to maintain long-term cultures of KS cells in vitro. In this study AIDS-KS–derived cells with characteristic spindle-like morphology were cultured with a growth factor (or factors) released by $CD4^+$ T lymphocytes infected with human T-lymphotropic virus type I or II (HTLV-I or HTLV-II) or with human immunodeficiency virus type 1 or 2 (HIV-1 or HIV-2). Medium conditioned by HTLV-II–infected, transformed lines of T cells (HTLV-II CM) contained large amounts of this growth activity and also supported the temporary growth of normal vascular endothelial cells, but not fibroblasts. Interleukin-1 and tumor necrosis factor–α stimulated the growth of the KS-derived cells, but the growth was only transient and these factors could be distinguished from that in HTLV-II CM. Other known endothelial cell growth promoting factors, such as acidic and basic fibroblast growth factors and epidermal growth factor, did not support the long-term growth of the AIDS-KS cells. The factor released by $CD4^+$ T cells infected with human retroviruses should prove useful in studies of the pathogenesis of KS.

KAPOSI'S SARCOMA DEVELOPS IN the form of multifocal lesions consisting of characteristic spindle-shaped cells in a stroma of proliferating abnormal vessels, fibroblasts, and infiltrating leukocytes. An indolent form of KS occurs in elderly males in Mediterranean and African countries (*1, 2*) and a more aggressive, glandular form of the disease occurs in younger Africans. An aggressive form of KS is also associated with HIV-1 infection, primarily in homosexual men (*3, 4*), and with immune suppression due to other causes (*5*). A direct transforming involvement of HIV-1 in the development of AIDS-associated KS (AIDS-KS) is unlikely because genomic sequences of the virus have not been detected in KS tissues (*6*). Furthermore, no other viruses, environmental factors, or genetic factors have been convincingly linked to any form of KS (*4, 6, 7*). To gain insight into the nature of KS cells and to search for possible new etiological agents, we developed procedures for establishing cells from AIDS-KS in culture.

A number of growth factors, including endothelial cell growth supplement (ECGS) and fibroblast growth factors (FGF), that were previously shown to stimulate or sup-

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Table 1. Stimulation of DNA synthesis in AIDS-KS cells and normal endothelial cells by known growth factors (*22*). AIDS-KS1 and AIDS-KS2 are two different cultures of spindle-like cells from KS lesions; HSF cells are normal human skin fibroblasts; KSF cells are fibroblasts from a KS lesion. To assess growth factor activity we measured levels of DNA synthesis ([^{3}H]thymidine uptake) (*22*). In addition to the results in the table we found that (concentrations tested shown in parenthesis), IL-2 (0.1 to 10% volume/volume), TGFβ (100 pg/ml to 20 ng/ml), NGF (1 to 50 ng/ml), GM-CSF (3 U to 100 U/ml), and heparin (3 to 90 μg/ml) did not stimulate DNA synthesis in either KS-derived or normal vascular endothelial cells. The experiments were repeated three times with similar results. ND, not done.

Growth factor*	Concentration per milliliter	Heparin (45 μg/ml)	Stimulation index†					
			AIDS-KS1	AIDS-KS2	BCE	H-UVE	HSF	KSF
ECGS	30 μg	–	1.3	1.1	5.7	20.8	1.3	1.9
		+	1.3	1.1	9.8	45.3	1.7	2.3
bFGF	50 ng	–	0.7	0.9	6.3	40.0	1.2	2.0
		+	0.5	0.6	4.8	32.4	1.0	1.7
EGF	50 ng	–	0.5	0.9	1.6	19.0	1.2	1.5
		+	0.5	0.6	1.2	14.9	1.1	1.4
PDGF	1 U	–	0.6	1.2	1.0	10.2	1.1	1.2
		+	0.7	0.9	0.7	8.6	1.0	1.0
IL-1	5 U	–	2.8	2.4	1.2	12.3	0.7	1.8
		+	2.0	2.0	1.3	6.8	0.5	1.1
TNFα	20 U	–	3.2	2.6	ND	1.5	ND	ND
		+	2.1	2.0	ND	1.2	ND	ND

*See (*9*). †See (*22*).

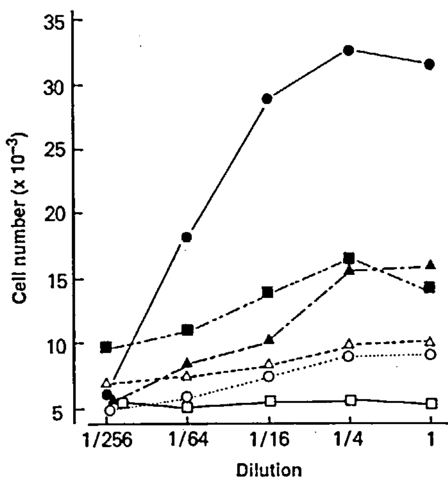

Fig. 1. Short-term growth of AIDS-KS cells with various concentrations of known growth factors and HTLV-II CM. Serum-free HTLV-II CM or the growth factors were serially diluted and added to AIDS-KS1 cells. Growth was monitored as described in Table 2. Cultures were initiated with 3×10^3 cells and cell number was determined on day 6 of culture (*22*). The experiments were repeated two times, with similar results. Symbols: ●, HTLV-II CM; ▲, IL-1 (5 U/ml); ■, TNFα (20 U/ml); △, γIFN (100 U/ml); ○, bFGF (50 ng/ml); and □, TNFβ (20 U/ml).

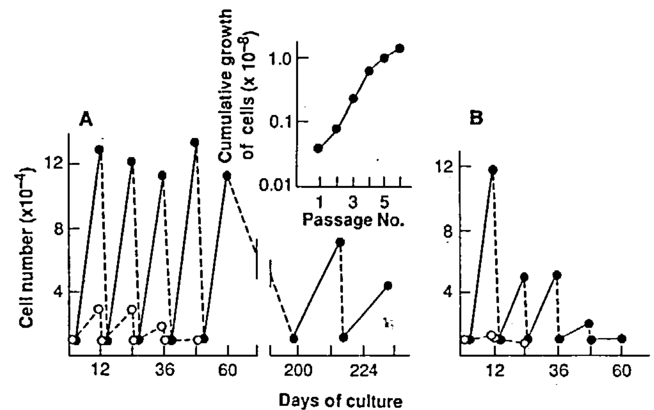

Fig. 2. Growth kinetics of (**A**) AIDS-KS1 cells and (**B**) early passage H-UVE in the presence (●) or absence (○) of HTLV-II CM. The AIDS-KS1 cells were subcultured continuously but the H-UVE cells showed a decreasing response after passage under the same conditions (*22*). (Inset) In vitro cumulative growth of KS-derived cells in culture medium supplemented with HTLV-II CM for more than six passages. The initial seeding density of the AIDS-KS1 cells was 0.5×10^6 cells per 75-cm^2 flask.

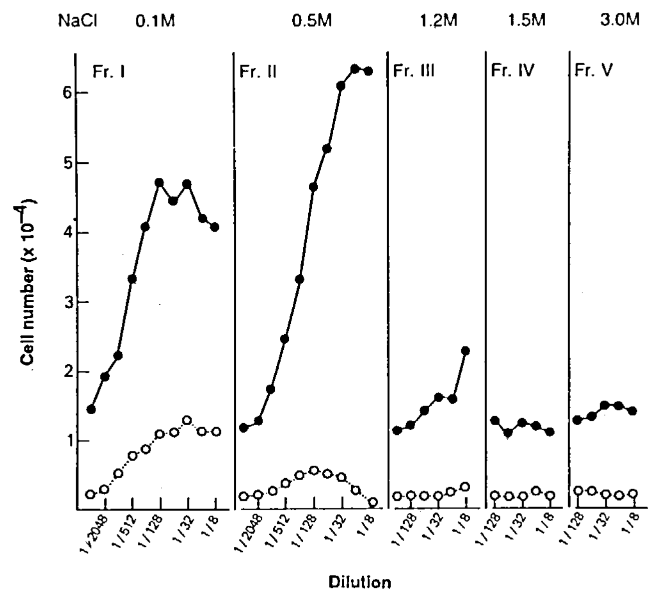

Fig. 3. Heparin affinity chromatography of HTLV-II CM. Heparin-Sepharose (Pharmacia) was equilibrated with 0.1*M* NaCl containing 0.01*M* tris-HCl (*p*H, 7.0). The HTLV-II CM (4000 U estimated with effective dose for 50% of maximum growth response of AIDS-KS1 cells) was applied in 0.1*M* NaCl with 0.01*M* tris-HCl buffer and was eluted in a stepwise manner with 0.5*M* NaCl (Fr. II), 1.2*M* NaCl (Fr. III), 1.5*M* NaCl (Fr. IV), and 3.0*M* NaCl (Fr. V), in addition to 0.01*M* tris-HCl alone. Each fraction was concentrated by ultrafiltration (YM-5, Diaflo), dialyzed against PBS, and assayed for growth stimulation of AIDS-KS cells (●) and H-UVE (○) at the indicated dilution (*22*). Recovery rate of activity was estimated with half-maximum effective dose. Basic FGF from bovine pituitary was used as positive control and identified in Fr. IV and Fr. V. Total recovery rate was 42% of the applied activity. Sixty-seven percent of the recovered activity in both assays was identified in Fr. I and 33% in Fr. II.

port the growth of many different types of cells [for reviews see (*8, 9*)] were screened for their ability to stimulate DNA synthesis in cells derived from a lung biopsy (AIDS-KS1) and from pleural effusion (AIDS-KS2) cells of AIDS patients with KS of the lungs. Although several of these factors stimulated bovine capillary endothelial (BCE) cells or human umbilical vein endothelial (H-UVE) cells (*10*), they had little or no affect on the AIDS-KS cells (Table 1). However, conditioned media (CM) from T cells infected with human retroviruses (*3, 11–13*), which were previously shown to

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produce several biological activities (*14*), contained activities that supported the growth of the AIDS-KS cells (Table 2). Since the highest level of activity was found in CM from an HTLV-II–infected cell line (HTLV-II CM), we used this source of CM for further analyses. As shown in Fig. 1, HTLV-II CM supported the growth of AIDS-KS cells in a dose-dependent manner. Although IL-1 and TNFα stimulated DNA turnover in AIDS-KS cells (Table 1), the maximum activity of these factors was significantly lower than that of HTLV-II CM over the range of concentrations tested (Fig. 1). Furthermore, HTLV-II CM maintained the growth of AIDS-KS cells for many months (average doubling time, 30 to 43 hours) (Fig. 2), whereas IL-1 and TNFα supported AIDS-KS cell growth for fewer than six passages. HTLV-II CM also supported the growth of normal endothelial cells (see H-UVE and BCE in Table 3). At optimum concentrations for AIDS-KS cells, HTLV-II CM had little effect on normal endothelial cells. Thus HTLV-II CM supported the growth of AIDS-KS cells for about 30 passages during 1 year, whereas H-UVE cells survived for less than 2 months under the same conditions (Fig. 2).

Comparison of the capacity of these various growth factors to support AIDS-KS and normal endothelial cells suggested that the factor (or factors) in HTLV-II CM differed from known endothelial cell growth factors. This was also suggested by the analysis of total and polyadenylated [poly(A)$^+$] RNA from three HTLV-II–infected cell lines that express high levels of the AIDS-KS factor as detected by molecular probes (*15, 16*) for several growth factors. By using molecular probes to known endothelial cell growth factors, little or no expression of mRNA was found for aFGF, bFGF, and TGFα. Similarly, no message could be found for IL-1β, IL-2, or B cell growth factor (BCGF), but mRNA was found for IL-1α, TNFα, TNFβ, GM-CSF, and TGFβ. However, these cytokines were not as effective as HTLV-II CM in supporting AIDS-KS cell growth.

Biophysical characterization of these activities showed distinct differences between known growth factors and the HTLV-II CM activity (Table 4). When HTLV-II CM was analyzed by heparin affinity column chromatography, the major activities that supported the growth of AIDS-KS cells and normal endothelial cells were identified in the flow through fraction I (Fr. I) and in the low salt eluate (Fr. II), indicating little or no heparin binding (*17*) (Fig. 3). In contrast, when bFGF was added to the column it was eluted in Fr. IV and Fr. V. Isoelectrofocusing analysis showed that the AIDS-KS cell growth factor had a peak isoelectric point (pI) of 7.3; TNFα, TNFβ, and IL-1α have significantly lower values (*18*) (Table 4). The activity in HTLV-II CM was heat stable for 30 min at 60°C, but was eliminated after 3 min at 100°C. The activity also disappeared when the CM was treated with trypsin and pronase, *p*H 7.4, at 37°C for 1 hour.

Analysis by gel filtration showed that the active component for AIDS-KS cells in HTLV-II CM had a molecular size of about 70 kD, whereas normal endothelial cell factor was 25 kD (Fig. 4A). Analysis by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions (Fig. 4B) indicated that the activity for KS cells appeared at ~30 kD, suggesting that the 70-kD form may represent an oligomer or carrier protein complex that can be dissociated to the monomer by SDS under reduced conditions. The activity for H-UVE cells was not recovered under the same conditions. Differences between HTLV-II CM and other growth factors were also suggested by im-

Table 2. Growth of AIDS-KS1 cells in the presence of CM from human retrovirus–infected cell lines. The CM was harvested at 4- to 5-day intervals (seeded at an initial density of 1×10^6 cells/ml). Supernatant fluids were clarified by centrifugation at low (2,000*g*) and high (45,000 rpm) speed for 1 hour, dialyzed against phosphate-buffered saline (PBS), passed through 0.45-μm Millipore filters, and stored at −20°C for at least 6 weeks before use. The CM were diluted 1:4 with RPMI 1640 before being tested (*22*). The experiments were repeated two times with similar results.

ECGS (30 μg/ml) and heparin (45 μg/ml)	Source of CM*						Medium control
	HTLV-I	HTLV-II	HIV-1 H9	HIV-2 Hut 78	H9	Hut 78	
7	11.4	20	16	15	9	8	5

*Results show number of viable cells ($\times 10^{-3}$) on day 6 after seeding in the presence of the indicated CM or growth factor. H9 is a cell clone (*3*) of probable HUT 78 origin which is used to grow HIV-1. HUT 78 (*23*) is a parental human leukemia CD4$^+$ T cell line used to grow HIV-2.

Table 3. Effects of various known growth factors and of HTLV-II CM on the growth of AIDS-KS cells (3×10^3 per well) and BCE and H-UVE cells (5×10^3 per well). Concentrations tested were: 1:8 dilution of HTLV-II CM; 30 μg/ml of ECGS with 45 μg/ml of heparin; 50 ng/ml of bFGF; and 5 U/ml of IL-1. The experiments were repeated three times with similar results.

Cell source	Factor tested*				
	HTLV-II CM	IL-1	bFGF	ECGS and heparin	Medium control
AIDS-KS1	17	11	3	5	4
AIDS-KS2	11	6.8	3.3	3	3.6
BCE	7	3	5	14	2.2
H-UVE	4	1.5	13	21	1.8

*Results show number of viable cells ($\times 10^{-3}$) on day 6 after seeding in the presence of the indicated CM or growth factor.

Table 4. A comparison of the biochemical and biological characteristics of a factor in HTLV-II CM that stimulates the growth of AIDS-KS cells to known lymphokines. The experiments were repeated two times with similar results.

Growth factor	Molecular size (kD)	Isoelectric point	Trypsin sensitivity	Heat stable at (°C)	Lectin binding	Promote growth of AIDS-KS cells	Augment growth of AIDS-KS cells in presence of hydrocortisone	Support growth of	
								Fibroblasts	H-UVE cells
TNFα*	17	<5.4	Sensitive	56°	Negative	Weak	Negative	Weak	Negative
TNFβ*	20	5.8	Resistant	55°	Positive	Negative	Negative	Negative	Negative
IL-1α*	17	5.0	Resistant	56°	Negative	Weak	Weak	Weak	Negative
AIDS-KS factor†	30	7.3‡	Sensitive	60°	~30% bound	Strong	Strong	Negative	Negative

*See (*18*). †Prepared with DEAE- and SP-cartridge (AMF Inc., Molecular Separation Division). ‡In two repeated experiments the results were 7.4 and 7.2.

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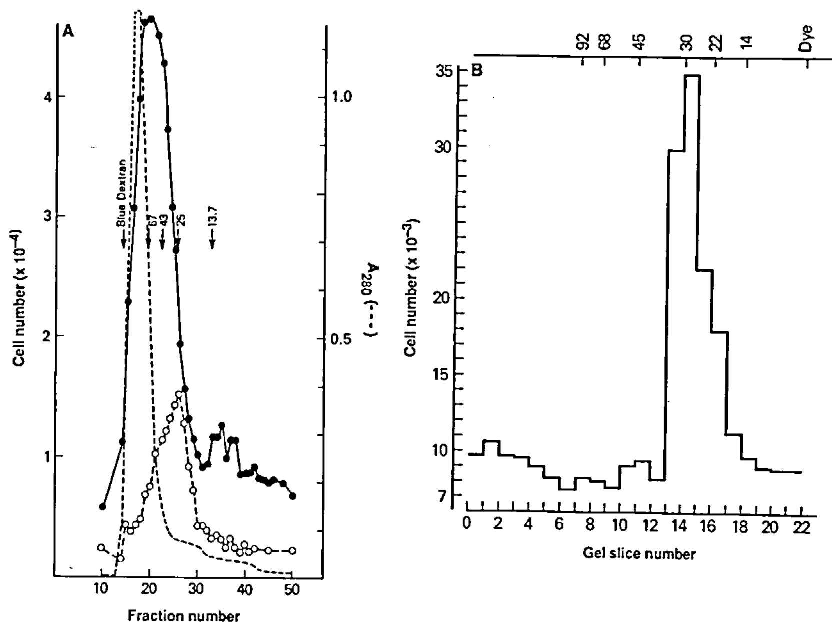

Fig. 4. Molecular size of AIDS-KS cell growth factor in HTLV-II CM determined by (A) gel filtration and (B) SDS-PAGE. **(A)** Concentrated (100×) serum-free HTLV-II CM (0.5 ml) was separated on a Sephadex G-75 column in PBS. One-eighth dilution of each fraction was assayed on AIDS-KS cells (●) and H-UVE cells (○) for growth promotion (*22*). Control IL-1 was identified in 20-kD through 15-kD fractions (17 kD in peak fraction) (data not shown). Standard markers (bovine serum albumin, 67 kD; ovalbumin, 43 kD; chymotrypsinogen, 25 kD; and ribonuclease, 13.7 kD) are indicated by arrows. **(B)** Profile of AIDS-KS cell growth promoting activity after SDS-PAGE. Semipurified HTLV-II CM (400 μl) was electrophoresed on 3 to 27% gradient polyacrylamide gels (1.5 mm thick) (Separagel). The sample buffer consisted of 1% SDS and 0.1*M* β-mercaptoethanol. The gels were sliced into 5-mm pieces, extracted with 1 ml of 50 m*M* NH_4HCO_3, and assayed for growth promoting activity on AIDS-KS1 cells (*22*). Each experiment was repeated two times with similar results.

munological analysis. For example, antibodies to IL-1 and TNFα did not neutralize the AIDS-KS cell growth promoting activity.

These studies indicate that at least two activities are produced by T cells infected by human retroviruses, one that predominantly supports the growth of AIDS-KS cells and another that is active for normal endothelial cells. They also indicate that the AIDS-KS cells and the normal endothelial cells used in our experiments differ with regard to their growth requirements. We have found that AIDS-KS cells themselves produce growth stimulating factors (*19*). Our data therefore suggest that some human retroviruses may induce the production of factors in vivo that influence the development of KS lesions. Vascularization has also been reported to occur after infection with HTLV-I (*20*), and KS has been reported in a patient with adult T cell lymphoma (*21*). The higher incidence of KS in patients infected with HIV-1 compared to patients infected with HTLV-I or HTLV-II could be due to the higher rate of replication of HIV-1 in large numbers of cells, which might result in the production of greater amounts of the initiating factor (or factors). In addition, immunodeficiency is more pronounced in individuals infected with HIV-1 than with HTLV, suggesting that this condition may also be important in the pathogenesis of KS. Confirmation of this possibility will depend on the development of specific assays for detection of the growth factor in patients. The system described here for establishing long-term cell cultures of AIDS-KS cells should facilitate further studies of the development of KS lesions.

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15. Nucleic acid probes for aFGF, bFGF, IL-1α, IL-1β, TNFα, TNFβ, IFNγ, GM-CSF, TGFα, TGFβ, IL-2, and BCGF (*16*) were used for evaluation. Northern blot technique was done according to T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), pp. 188–206. The antisense oligomers were labeled by incubation with [^{32}P]adenosine triphosphate and T4 polynucleotide kinase. These probes were added to the same solution (1×10^6 cpm/ml) and the hybridization was continued at 37°C. The membranes were subsequently washed with 2× SSC + 1% SDS for 25 min at room temperature, 20 min at 45°C, and 20 min at 65°C, then air-dried and exposed for 3 days. For RNA analysis for IFNγ and IL-2, blots were hybridized to their ^{32}P-labeled nick-translated probes at 37°C, then washed three times with 2× SSC containing 0.5% SDS for 30 min at 65°C, twice with 1× SSC containing 0.5% SDS for 30 min at 65°C, and twice with 0.1× SSC containing 0.5% SDS for 20 min at 65°C, then air-dried and exposed for 3 days.
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22. Stimulation of DNA synthesis and cell number were measured as follows: KS cells (3×10^3 cells per well) or H-UVE or BCE cells (5×10^3 cells per well) were incubated in 24-well plates with 15% fetal calf serum (FCS) in RPMI 1640 medium in the absence or presence of test factors or CM. Plating efficiency was approximately 70% for each KS cell culture and 50% for H-UVE and BCE cells. Medium and specimens were replaced at day 3 (KS cells) and on day 2 (H-UVE and BCE cells) with 1 μCi per well of [^{3}H]thymidine and incubated overnight. The stimulation index was calculated as: [^{3}H]thymidine uptake in the presence of growth factors divided by [^{3}H]thymidine uptake in control medium. The cell number was determined with a Coulter particle counter after trypsinization of cells on day 6 of culture. For fibroblast growth determination, the cells (3×10^3 per well) were incubated with RPMI 1640 medium supplemented with FCS (10%) in the presence or absence of test CM in 24-well plates. Cells were incubated overnight with [^{3}H]thymidine (1 μCi per well) on day 4 of culture; uptake was measured on day 5. The AIDS-KS cells were subcultured and maintained on gelatinized plastic ware with the use of complete culture medium with HTLV-II CM (20% v/v). The H-UVE and BCE cells were also cultured in the gelatinized flasks with ECGS (30 μg/ml) and heparin (45 μg/ml) in addition to the complete culture medium.
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Angiogenic Properties of Kaposi's Sarcoma–Derived Cells After Long-Term Culture in Vitro

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Cells derived from lung biopsies and pleural effusions from AIDS patients with Kaposi's sarcoma (KS) of the lungs were established in long-term culture with the aid of conditioned medium from HTLV-II–transformed T cells (HTLV-II CM). These AIDS-KS cells were similar to the so-called spindle cells in KS lesions and had some of their features. They produced factors that supported their own growth (autocrine) and the growth of other cells (paracrine), including umbilical vein endothelium and fibroblasts. That the AIDS-KS cells also expressed potent angiogenic activity was demonstrated by the chorioallantoic membrane assay and by subcutaneous inoculation of AIDS-KS cells into nude mice, which resulted in the development of angiogenic lesions composed of mouse cells and showing histological features similar to those of human KS lesions. These data suggest that AIDS-associated KS and possibly other types of KS may be initiated by signals that induce the growth of particular cells (spindle cells of lymphatic or vascular origin) and the expression of autocrine and paracrine activities.

Kaposi's sarcoma was first described more than 100 years ago (*1*). The disease was rare in Caucasians and usually limited to elderly men, although an aggressive form was subsequently found to occur in young people in Africa. This latter form of KS has several features in common with the KS associated with infection by human immunodeficiency virus type 1 (HIV-1) (*2, 2a*) and the KS that occurs in transplant recipients (*3*) and other patients receiving immunosuppressive therapy (*4*). KS lesions have a complex histology characterized by abnormal vascularization and the presence of proliferating endothelium and "spindle" cells, fibroblasts, and infiltrating leukocytes (*5*). Studies of the origin of the KS have been inconclusive, in part because of the complex nature of the lesions but also because of the lack of appropriate culture systems in vitro.

Several features of the lesions of KS suggest that they represent polyclonal proliferations rather than true tumors. Genetic factors (*2*) as well as certain herpesviruses (*6*) and drugs (*2*), acting independently or in concert, have all been suggested as contributing to KS. However, none of these factors have convincingly been linked to the pathogenesis of the disease (*7, 8*). Although there is a clear correlation between HIV-1 infection and the aggressive, epidemic form of AIDS-KS, no genomic sequences of HIV-1 or of any other virus have been detected in KS tissues (*8*). We have now characterized AIDS-KS cells cultured in the presence of conditioned medium (CM) from HTLV-II–infected and –transformed T cell lines (*9*), and have obtained data that are of relevance to the understanding of the origin and biology of KS.

Six KS cell lines were used: AIDS-KS1, AIDS-KS3, and AIDS-KS4 were initiated from lung biopsies; the other lines were derived from pleural effusions from male AIDS patients with lung KS (*9*). Most of the cells had some morphologic features characteristic of KS "dendritic" spindle cells (Fig. 1) (*5*). Ultrastructural examination indicated that they were undifferentiated proliferating cells with prominent smooth (SER) and rough endoplasmic reticulum (RER), gap junctions and "foot" junctions, vermiform and branched mitochondria (Figs. 2), and often prominent, clustered, interchromatin granules in the nucleus. The appearance of the RER and SER was suggestive of well-developed secretory functions. The cells lacked the Weibel-Palade bodies (*10*) that are characteristic of cultures of normal vascular endothelial (H-UVE) cells but are not usually seen in spindle cells of primary KS tissues (*5*). No specific chromosomal abnormalities have been described in cells from KS tissues (*8*), and the cultured AIDS-KS cells also had a normal, human male diploid karyotype (46XY). The most common variation observed was a missing Y chromosome, which frequently occurs in cultured cells.

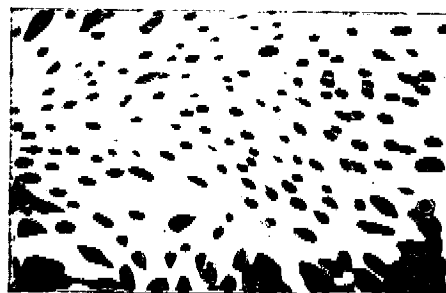

Fig. 1. Long-term cultured confluent AIDS-KS cells stained with Wright-Giemsa. Cells obtained from tissue biopsies from KS lesions by digestion with trypsin (0.05%) and EDTA (0.5 m*M*), as well as cells obtained from pleural effusion from AIDS patients, were grown in a growth medium (RPMI 1640, 15% fetal calf serum) supplemented with HTLV-II CM in 96-well plates by limiting dilution methods.

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