

AIDS-Kaposi's Sarcoma-Derived Cells Express Cytokines with Autocrine and Paracrine Growth Effects

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When grown in vitro, cells from Kaposi's sarcoma lesions of AIDS patients (AIDS-KS cells) constitutively release several growth promoting activities. When inoculated into nude mice, the AIDS-KS cells induce a KS-like lesion of mouse origin. Here it is shown that the AIDS-KS cells express messenger RNA for a complex mixture of cytokines that correlate with several of the biological activities of these cells. Basic fibroblast growth factor, which is a potent angiogenic factor, and interleukin-1 messenger RNAs are expressed at very high levels and seem to account for a large proportion of the activities, since their corresponding proteins are released in biologically active form into the culture media where they induce autocrine and paracrine growth effects.

KAPOSI'S SARCOMA (KS) IS A MULTIFOCAL proliferative lesion of unknown etiology and unclear pathogenesis involving cutaneous and visceral tissues (1). The KS that is frequently associated with infection with the human immunodeficiency virus, HIV, has a similar histology to other forms of KS including the classical, indolent form (2-6). After long-term growth in vitro, KS cells from AIDS patients (AIDS-KS cells) (5, 6) release activities that not only promote their own growth (autocrine activities), but also promote the growth of normal human endothelial cells and fibroblasts (paracrine activities) (6). The AIDS-KS cells produce an interleukin-1 (IL-1)-like activity, and activities promoting chemotaxis of normal endothelial cells. AIDS-KS cells, or their products, can also trigger angiogenic responses in the chick chorioallantoic membrane and can induce a KS-like lesion in nude mice (6).

To correlate these activities to specific gene expression we studied the production of cytokine messenger RNA (mRNA) by AIDS-KS cells, using AIDS-KS cells that had been grown in the presence of conditioned medium (CM) from HTLV-II-infected lymphocytes (5, 6). We also studied mRNA expression by normal endothelial and other control cells under similar conditions and at various intervals during culture. Control studies were also performed on AIDS-KS cells washed twice in phosphate-buffered saline (PBS) and grown with the same media used for normal endothelial cells (ECGS and heparin).

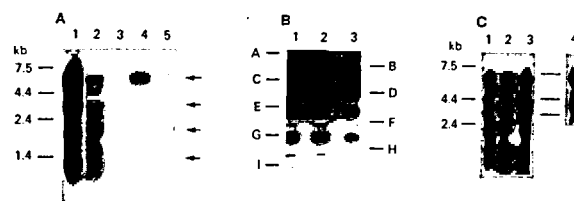
The expression of mRNA for basic fibro-

blast growth factor (bFGF) and endothelial cell growth factor (ECGF, also known as acidic FGF or aFGF) (7), was of interest because both cytokines induce endothelial cell growth in vitro and angiogenesis in vivo (7). RNA blot analysis with oligonucleotide probes for bFGF mRNA revealed specific bFGF transcripts in the AIDS-KS cell cultures and in the control cells [SK-HEP-1, a human hepatoma cell line, and BAE, bovine aortic endothelial cells (8)]. In contrast, little or no expression was detected in long-term cultures of normal human umbilical vein endothelial cells (H-UVE) (Fig. 1A). Two bFGF transcripts, of 7 and 3.7 kb, were reported previously in several cell types (9-11). However, we detected this mRNA pattern only in BAE cells and found at least

four transcripts, of 7, 3.7, 2.2, and 1.4 kb, in all the human cell types tested (Fig. 1A). AIDS-KS cells produced three to six times more bFGF mRNA than SK-HEP-1 and BAE cells, and at least 100 times more than H-UVE cells (Fig. 1B). Repeated hybridization under high stringency conditions, with several bFGF clones obtained from a library constructed from an AIDS-KS cell clone (12), confirmed these results. Moreover, sequence analysis of several selected cDNA clones showed identity to the published coding sequences of bFGF (12). No amplification or major rearrangement of the bFGF gene was observed in AIDS-KS cells that could account for the high level of expression. Because of the recent isolation of a bFGF-related gene (KS oncogene) from one of several KS tissues tested (13), and because of its identity with the *hst* oncogene (14), we also examined the AIDS-KS cells for expression of *hst* (15), but found no RNA expression or major rearrangement of this gene. The *hst* (KS oncogene) is therefore unlikely to account for the biological properties of the AIDS-KS cells. The mRNA for ECGF (or aFGF) was expressed at much lower levels by the AIDS-KS cells [mRNA species of 7, 4.2, and 2.8 kb (16)], and was not detected in normal endothelial cells (Fig. 1C).

The AIDS-KS cells expressed high levels of IL-1 β mRNA (1.8 kb) but low levels of IL-1 α mRNA (2.2 kb) (Fig. 2A) (17). These results were of interest because exogenously added IL-1 promoted transient growth of AIDS-KS cells (5, 6). Normal endothelial cells (H-UVE and BAE) did not

Fig. 1. (A) RNA blot analysis of bFGF mRNA from AIDS-KS and control cells. Total cell RNA (20 μ g) was used for samples in lanes 1 to 4. Lanes: 1, AIDS-KS3; 2, SK-HEP-1; 3, H-UVE; 4, BAE cells. Poly(A)⁺ RNA (5 μ g) was used for lane 5: PBMC (peripheral blood mononuclear cells). Total RNA and poly(A)⁺ RNA were prepared as described (30), subjected to electrophoresis on a 1% agarose gel containing 2.2M formaldehyde, and transferred to Hybond-N membrane (Amersham) by electroblot. Blots were prehybridized at 37°C in 2 \times SSC, 0.7% SDS, 30% formamide, 5 \times Denhardt's solution, 5% dextran sulfate, denatured salmon sperm DNA (100 μ g/ml), and yeast tRNA (50 μ g/ml) (30). Three antisense oligodeoxynucleotide probes (31) were added to the same solution (1 \times 10⁶ per milliliter) and the hybridization continued for 16 hours at 37°C. The membranes were subsequently washed with 2 \times SSC containing 1% SDS at room temperature for 25 min, and at 45°C and 65°C for 20 min each. They were air-dried and exposed to Kodak XAR film with intensifying screens. Size markers are RNA-leader (BRL). The specific transcripts are indicated by arrows. (B) Slot blot analysis of bFGF RNA from AIDS-KS and control cells. Lanes 1, 2, and 3: 33.3, 12.5, and 4.6 μ g, respectively, of the total RNA from AIDS-KS cell cultures. [A to E are AIDS-KS3, -KS1, -KS2, -KS4, and -KS5, respectively, as described in (6); F is LPS-activated PBMC; G, SK-HEP-1; H, H-UVE; and I, *Escherichia coli*]. Hybridization was carried out with three oligonucleotide probes [bFGF-1, -2, -3 (31)], mixed together as described in (A). The autoradiographs were estimated by densitometry after overnight (ON) exposure. (C) Expression of ECGF mRNA by AIDS-KS cells and normal endothelial cells and fibroblasts. Lanes: 1, total RNA (20 μ g) from AIDS-KS3 cells; 2, poly(A)⁺ RNA (4.3 μ g) from AIDS-KS3 cells; 3 and 4, total RNA from H-UVE (20 μ g) and human skin fibroblasts (HSF) (15 μ g), respectively. HSF cells, known to express ECGF, were included as a positive control. Hybridization was carried out with a mixture of ECGF-1 and ECGF-2 oligonucleotide probes derived from the published human ECGF-cDNA sequence (32): ECGF-1 (36-mer) nucleotides 118-154 and ECGF-2 (39-mer) nucleotides 240-278. For materials and methods, see (A).



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express detectable levels of IL-1 α or - β mRNAs (Fig. 2A). However, H-UVE cells can express low levels of IL-1 mRNA, depending on the cell source and culture conditions (5). Expression of IL-1 β mRNA by the AIDS-KS cells was at least 100 times greater in the AIDS-KS cells than in the H-UVE cells in this determination (Fig. 2B). However, we found no rearrangement or amplification of the IL-1 β gene.

Granulocyte-monocyte colony-stimulating factor (GM-CSF) mRNA was expressed by AIDS-KS cells but not by H-UVE or BAE cells (18). Two major RNA species were detected in the AIDS-KS cells and in an HTLV-I-transformed CD4⁺ cell line used as a positive control (19). Only the expression of the smaller transcript of GM-CSF (1 kb) correlated with the presence of the biological activity in the cell supernatant

(20). AIDS-KS and H-UVE cells, but not BAE cells, expressed mRNA for transforming growth factor- β (TGF- β) (2.5 kb) (21), while platelet-derived growth factor B (PDGF-B) mRNA (~3.7 kb) (22) was expressed in all the cells tested: AIDS-KS, H-UVE, and BAE cells. No expression of mRNA for other cytokines, including transforming growth factor- α (TGF- α), angiogenin, monocyte-colony-stimulating factor (M-CSF), tumor necrosis factor- α and - β (TNF- α and - β), was found in AIDS-KS cells (23) (Table 1). The difference in expression of growth factor mRNAs between AIDS-KS and normal endothelial cells was not dependent on time in culture or on the presence of different growth supplements. In fact, early passages of AIDS-KS and H-UVE culture cells grown in identical and parallel conditions for 5 weeks, and repeat-

edly analyzed after week 1 and up to week 5, expressed the same specific mRNAs, as described above, after long-term cultures (~1 year) and under optimal growth conditions. No induction of cytokines was detected in H-UVE cells grown in HTLV-II CM. Furthermore, H-UVE cells were unable to grow more than 1 to 2 weeks in HTLV-II CM (5).

Since bFGF and IL-1 were expressed at much higher levels in the AIDS-KS cells compared to control cells, and in view of their known biological activities, we further focused on these two cytokines. Radioimmunoprecipitation assays (RIPA) were performed with cell extracts and supernatants from metabolically labeled AIDS-KS cells. Both bFGF and IL-1 were synthesized in considerable quantities by the AIDS-KS cells (Fig. 3, A to C). In AIDS-KS cell extracts, three protein species of approximately 17, 25, and 27 kD were recognized by a polyclonal antiserum to a synthetic peptide of bovine bFGF (24) (Fig. 3A, lane 1), whereas H-UVE cell extracts contained no detectable protein (Fig. 3A, lane 3). Preincubation with purified bFGF (Fig. 3B) eliminated all three bFGF-bands. We did not determine whether the 27- and 25-kD species represent alternative forms of post-translational modifications of the previously described 17-kD bFGF species (7, 8). However, a 25-kD form of bFGF was recently described in guinea pig brain together with the 17-kD band (25). Comparison of the levels of bFGF production in AIDS-KS cells and control cells was in agreement with the mRNA expression levels (Fig. 3C). In fact, AIDS-KS cells produced 100 times more bFGF than H-UVE cells, 10 to 15 times more than BAE cells, and 5 times more than SK-HEP-1 cells (a known high producer of bFGF). Attempts to immunoprecipitate any of the bFGF species from the cell supernatant of AIDS-KS cells were unsuccessful, although a potent bFGF-like activity was present in AIDS-KS supernatant (6). It is possible that our inability to detect secreted bFGF could be due to the low sensitivity of the technique for proteins below the concentration of 0.5 μ g/ml. In fact, bFGF is already active at concentrations of 0.5 to 1.0 ng/ml; therefore, the biological assay is more sensitive.

The AIDS-KS cells produced high levels of IL-1 but the H-UVE cells produced low or nondetectable amounts (Fig. 3, D and E). IL-1 was present in extracts prepared from AIDS-KS cells as a ~32-kD species as previously described for other cell types (26), whereas in the supernatant (CM), ~23- and ~27-kD forms were detected (Fig. 3, D and E). Proteolytic processing of the ~32-kD species may result in the release of these proteins, as shown by others (26). The

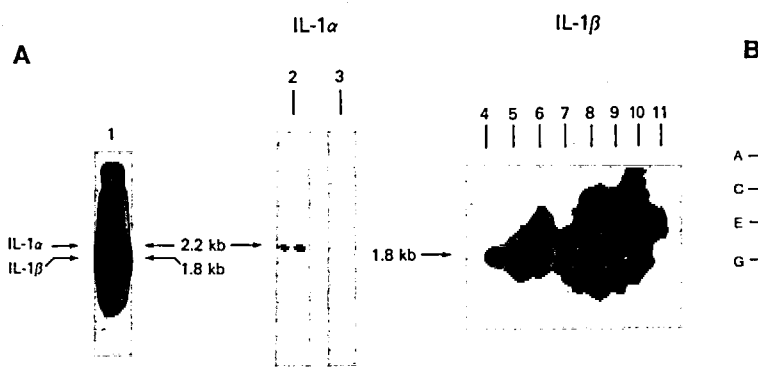


Fig. 2. (A) Expression of IL-1 α and IL-1 β mRNA from AIDS-KS and other human cells. Lanes: 1, total RNA (15 μ g) from LPS-activated PBMC was used as a positive control and hybridized to both IL-1 α and IL-1 β probes; 2 and 3, IL-1 α probe [poly(A)⁺ RNA (5 μ g) from AIDS-KS3 cells and total RNA (20 μ g) from H-UVE cells, respectively]; 4 to 11, IL-1 β probe; 5, poly(A)⁺ RNA (5 μ g) from AIDS-KS cells; 4, 6, and 11, total RNA (15 μ g) from AIDS-KS cells (AIDS-KS3, -KS1, -KS4, -KS5, and -KS6), H-UVE cells, respectively. Hybridizations were carried out as described in Fig. 1A with IL-1 α (48-mer) and IL-1 β (48-mer) oligonucleotide probes, derived from nucleotides 769-816 of IL-1 α and 763-810 of IL-1 β cDNA published sequences, respectively (17). BAE cells did not express IL-1 mRNAs. (B) Slot blot analysis of IL-1 β mRNA from AIDS-KS and control cells. Lanes: 1, 2, and 3, 33.3, 12.5, and 4.6 μ g of total RNA from AIDS-KS cells [A to E are AIDS-KS3, -KS1, -KS2, -KS4, -KS5, respectively]; F, LPS-activated PBMC; G, H-UVE and H, *E. coli*. Hybridization was carried out with IL-1 β oligonucleotide probe described in (A). Densitometry was performed after ON exposure.

Table 1. Growth factor mRNA expression by AIDS-KS-derived cells and normal cells. Total RNAs (15 to 20 μ g) were extracted as described (30) from each cell type and analyzed by standard RNA blot technique. The results are a mean of at least three independent analyses. AIDS-KS and H-UVE cells that had been grown in vitro for up to 1 year (5, 6) were harvested for RNA extraction at ~80% confluency. The relative expression of RNA is given as: -, negative; \pm , low signal not consistently detected; ++, 5 to 10 times the minimal detectable signal; +, +++, 10 to 50 times +; and +++++, >50 times +. None of the cells showed detectable mRNA for TGF- α , M-CSF, TNF- α , TNF- β , or angiogenin. Short-term cultures (5 weeks) of AIDS-KS cells grown in RPMI 1640 supplemented with HTLV-II-derived CM (5), or with ECGS and heparin (5), did not show differences in RNA expression. The same results were also obtained for AIDS-KS and H-UVE cells when contemporary cultures, under identical conditions (growth medium and time in culture), were initiated and analyzed at week 1, 2, 3, 4, and 5 of culture. The same results were also obtained with different preparations of media and fetal calf serum.

RNA source	Cytokine mRNA						
	bFGF	ECGF	IL-1 α	IL-1 β	GM-CSF	TGF- β	PDGF- β
AIDS-KS cells	++++	+	+	+++	++	+(+)	+
Normal endothelial cells (H-UVE)	\pm	-	-	\pm	-	++	++

RIPA showed that the AIDS-KS cells produced the highest levels of IL-1 (15 to 25 times more than H-UVE and at least 100 times more than BAE cells) (Fig. 3E).

To determine whether the KS-derived IL-1 and bFGF molecules were biologically active, we used specific antibodies to bFGF and IL-1. When CM from AIDS-KS cells

was incubated with the bFGF antiserum (24), the induction of H-UVE cell growth was specifically blocked (Fig. 4A), indicating that a bFGF-like molecule (5) is released by the AIDS-KS cells in a biologically active form. Experiments with AIDS-KS cells and the same bFGF antiserum (24) gave similar results (20). Furthermore, when we repeated the experiment using affinity-purified neutralizing antibodies to the native bovine brain bFGF (27), AIDS-KS cell growth was clearly reduced (Fig. 4B). In this experiment, AIDS-KS cells were grown with AIDS-KS CM and incubated with either antibody to native bFGF (27) or nonimmune serum. Control media treated with the antibody to bFGF were also examined under the same conditions. Both the AIDS-KS cell growth induced by the AIDS-KS CM and the basal cell proliferation were reduced only by the antibodies to bFGF. We showed previously (5) that bovine bFGF has only a small effect on AIDS-KS cell growth. This could be due to presaturation of the receptor by the endogenous bFGF as shown for other overexpressed growth factors (TGF- α and ECGF) in different cell systems (28), or to differences in the bFGF-like molecule (or molecules) released by these cells as compared to the bovine bFGF, or, finally, to differences in the bFGF-receptors on AIDS-KS cells.

The IL-1-like activity in the AIDS-KS CM (6) was also related to the release of IL-1 by AIDS-KS cells. The addition of antibodies to IL-1 (29) to AIDS-KS cells grown either with AIDS-KS CM or control media specifically inhibited, but did not abolish, cell proliferation (Fig. 4C).

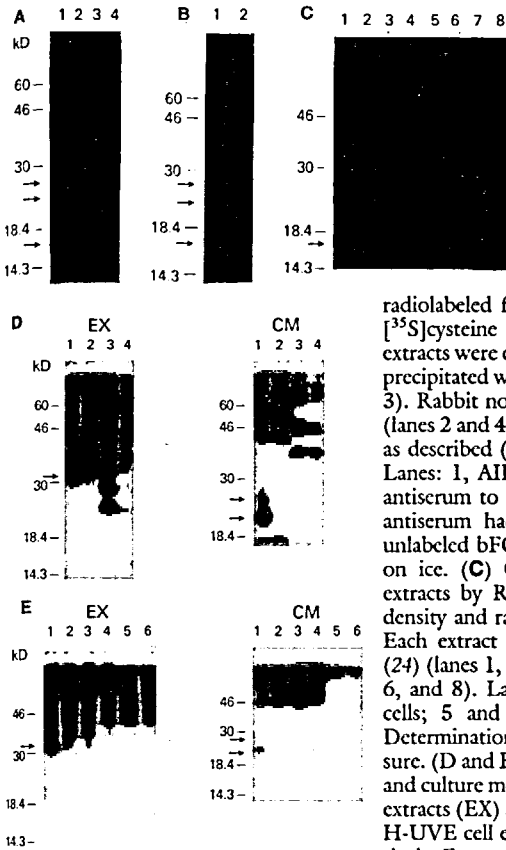
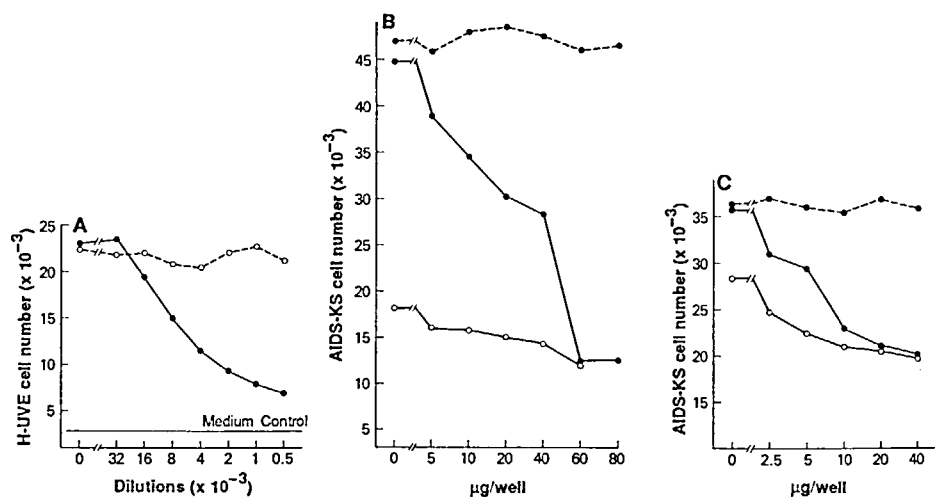


Fig. 3. (A to C) Analysis (RIPA) of bFGF in extracts derived from AIDS-KS cells, normal cells (H-UVE, BAE), and an additional control cell line (SK-HEP-1). (A) Lanes: 1 and 2, AIDS-KS-derived cell extracts; 3 and 4, H-UVE-derived cell extracts. AIDS-KS and H-UVE cells were grown in culture as described (5, 6). Cells (~80 confluency) were rinsed twice in PBS, incubated at 37°C for 2 hours in cysteine- and methionine-free RPMI 1640 with 1% dialyzed FCS (dFCS), and then radiolabeled for 6 to 8 hours in similar media containing [³⁵S]cysteine and methionine (200 μ Ci/ml each). Cellular extracts were cleared with normal rabbit serum and immunoprecipitated with rabbit antiserum to bFGF (24) (lanes 1 and 3). Rabbit nonimmune IgG was used as a negative control (lanes 2 and 4). Proteins were separated by 15% SDS-PAGE as described (33). Arrows show specific protein bands. (B) Lanes: 1, AIDS-KS-derived cell extracts precipitated with antiserum to bFGF (24); 2, the same extract in which the antiserum had been competed with 0.5 μ g of purified unlabeled bFGF (bovine pituitary bFGF, ABI) for 1 hour, on ice. (C) Quantitative determination of bFGF in cell extracts by RIPA. Cells (0.5×10^6) were seeded at low density and radiolabeled (100 μ Ci/ml) as described in (A). Each extract was precipitated with the specific antiserum (24) (lanes 1, 3, 5, and 7) or with control serum (lanes 2, 4, 6, and 8). Lanes: 1 and 2, AIDS-KS3; 3 and 4, H-UVE cells; 5 and 6, BAE cells; 7 and 8, SK-HEP-1 cells. Determination by densitometry was done after ON exposure. (D and E) Analysis (RIPA) of IL-1 in cell extracts (EX) and culture media (CM). (D) Lanes: 1 and 2, AIDS-KS3 cell extracts (EX) and culture media (CM), respectively; 3 and 4, H-UVE cell extracts (EX) and culture media (CM), respectively. For procedures, see (A). A rabbit antiserum to human IL-1 was used (29) (lanes 1 and 3). Rabbit preimmune IgG was used as a negative control (lanes 2 and 4). (E) Quantitative determination of IL-1 in cell extracts (EX) and culture media (CM) by RIPA. Lanes: 1 and 2, AIDS-KS cells; 3 and 4, H-UVE cells; 5 and 6, BAE cells. For procedures, see (C). Antiserum to human IL-1 (29) was used for lanes 1, 3, and 5, and preimmune IgG for the control lanes 2, 4, and 6.

Fig. 4. (A) Inhibition of the AIDS-KS CM-induced growth of normal vascular endothelial cells by antiserum to bFGF. H-UVE cells (1×10^3 cells per well) were grown with AIDS-KS derived CM (●) (dilution 1:2) or with ECGS (30 μ g/ml) and heparin (45 μ M/ml) (○), as described (5, 6). The same CM were treated by ON incubation at 4°C with several dilutions of antiserum to bFGF (24) and then added to the cell cultures. The cell number was determined (Coulter counter) after trypsinization of cells on day 6 of culture. The control medium (RPMI 1640 and 15% FCS) did not induce H-UVE cell proliferation. An additional control in which AIDS-KS-derived CM was treated with nonimmune rabbit IgG did not show reduction of the H-UVE cell proliferation. (B) Inhibition of AIDS-KS cell growth by antibodies to bFGF. AIDS-KS cells (3×10^3 cells per well) were incubated with 10% FCS in RPMI 1640. AIDS-KS-derived CM (dilution 1:2) was treated by ON incubation at 4°C with several concentrations of antibodies to bFGF (27) (from 5 to 80 μ g of purified IgG) (●, —) or with nonimmune serum (●, - -), control medium (10% FCS in RPMI 1640) (○) was treated with antibodies to bFGF. The treated media were then added to the cells. The same concentrations of antibodies were added to the wells every other day; cell number was determined (Coulter counter) after trypsinization of cells on day 6. (C) Inhibition of AIDS-KS cell growth by antibodies to IL-1. See (B) for procedures. Initial cell seeding was 1×10^4 and



the AIDS-KS CM was diluted 1:8. Antibodies to IL-1 (29) were used at concentrations of 2.5 to 40 μ g of purified IgG per well. (●, —), AIDS-KS CM treated with antibodies to IL-1; (●, - -), AIDS-KS CM treated with preimmune antibodies; (○, —), control medium treated with antibodies to IL-1. All blocking experiments (A, B, and C) were repeated a minimum of three times, each in duplicate with no significant variations in the result.

Thus AIDS-KS cells synthesize and release relatively large amounts of biologically active bFGF-like molecules and IL-1 that can induce self-proliferation. In addition to this autocrine growth effect, the released bFGF-like molecules also induce proliferation of normal endothelial cells (paracrine). In the human KS lesion, the proliferation of spindle cells, endothelial cells, and fibroblasts is associated with angiogenesis and inflammatory cell infiltration (2-6). Interaction among endothelial cells, fibroblasts, and cells of the immune system induced by the bFGF-like molecule and IL-1 (and possibly by the other cytokines expressed by AIDS-KS cells) could be responsible for the lesion induced in the nude mouse by the AIDS-KS cells (6). The data presented here thus support the idea (5, 6) that, in humans, the putative novel growth factor released by CD4⁺ T cells infected with one of the human retroviruses (5) initiates cellular events that lead to the production of cytokines and consequent cell growth and histologic changes relevant to the pathogenesis of AIDS-associated KS.

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- bovine cells may reflect heterogeneity of bFGF-related mRNA in different cells derived from different species, or may be due to the presence of precursor transcripts (such as the 6.8- to 7-kb band) containing untranslated flanking regions, or to the use of different initiation or termination sites of transcription (9-11).
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Dynamic Expression Pattern of the *myc* Protooncogene in Midgestation Mouse Embryos

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The *c-myc* protooncogene in mouse embryos was shown by RNA in situ hybridization to be preferentially expressed in tissues of endodermal and mesodermal origin. Most organs developing from the ectoderm, such as skin, brain, and spinal cord, displayed low levels of *c-myc* RNA. The thymus represented the only hematopoietic organ with high *c-myc* expression. In organs and structures strongly hybridizing to *c-myc* probes, for example the fetal part of the placenta, gut, liver, kidney, pancreas, submandibular glands, enamel organs of the molars, and skeletal cartilage, the level of expression depended on the stage of development. Expression was observed to be correlated with proliferation, particularly during expansion and folding of partially differentiated epithelial cells.

THE PRODUCT OF THE *c-myc* PROTOONCOGENE is thought to mediate the action of growth factors in many cell types (1). In general, *c-myc* expression seems to confer proliferation competence to cells and is switched off during terminal differentiation in differentiated leukemic cells of various lineages (2). To study the effect of *c-myc* on normal development and tumor formation, *c-myc* gene constructs have been introduced into the germ line of mice, allowing tissue-specific or generalized expression of its product (3). No gross developmental disturbances were observed in these transgenic animals, although tumor formation was increased in specific tissues or nonspecifically.

To study *c-myc* in normal development

Zimmerman et al. (4) observed *c-myc* expression in whole embryos beginning from day 15 of embryonic development, but specific tissues were not analyzed. In newborn mice, generalized expression of *c-myc* in different tissues was observed. In human embryos, Pfeifer-Ohlson et al. (5) investigated *c-myc* expression by RNA in situ hybridization with ¹²⁵I-labeled DNA probes. The *c-myc* expression was low in embryos at 3 to 4 weeks. However, embryos of 5 to 10 weeks displayed a high hybridization signal over

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