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25. The membranes were composed of asolectin, and the aqueous phases were 100 mM NaCl, 5 mM dimethylglutarate acid, 2 mM $MgCl_2$, and 1 mM EDTA. The pH was 6.8 in the cis compartment and 7.2 in the trans compartment. TNF was added to a final concentration of 12.5 ng/ml.
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28. "Solvent-free" membranes were prepared as described (*23*) with squalene (Sigma) or squalane (Fluka) to coat a hole (100 to 200 μm in diameter) in a Teflon partition. Monolayers were spread from mixtures of soybean phosphatidylethanolamine (40%), soybean phosphatidylcholine (40%), and bovine phosphatidylserine (20%) (Avanti, Pelham, AL). Occasionally this lipid mixture was mixed 1:1 with asolectin (*24*). Capacitance measurements were used to monitor bilayer formation from the apposition of the two monolayers. After membrane formation, the conductance ($g = I/V$) of the unmodified membrane was ohmic and was ~5 to 10 pS. All membranes used exhibited low conductance and were stable to voltages of ±100 mV for at least 10 min before the addition of TNF. The aqueous phases usually included 100 mM NaCl, 5 mM dimethylglutaric acid (pH 4.5) or 5 mM tris (pH 7.2) as buffer, 2 mM $MgCl_2$, and 1 mM EDTA. Voltage-clamp conditions were used; a battery-driven stimulator was used to apply voltages and a Keithley 427 current amplifier was used to measure current, which was recorded on an oscilloscope and a chart recorder. The cis compartment, to which TNF was added, is defined as ground. Voltages refer to the trans compartment, opposite the TNF-containing side and analogous to the cytosol of a target cell. In ion-selectivity experiments, salt gradients were imposed across the membrane, and the zero-current reversal potential E, where $I = g(V - E)$, was measured. Silver/silver chloride electrodes were used to connect the solutions to the electronics, and 3 M KCl/agar salt bridges were used in salt gradient experiments. Human recombinant (clinical grade) TNF was from Genentech (South San Francisco, CA); similar results were obtained with natural TNF from Calbiochem (San Diego, CA).
29. Same buffer as in Fig. 2A [see (*25*)], but pH 7.2 cis, pH 7.2 trans, and +100 mV. The lipid was a mixture of asolectin (33%), soybean phosphatidylethanolamine (27%), soybean phosphatidylcholine (27%), and bovine phosphatidylserine (13%); final concentration of TNF was 100 ng/ml.
30. Data are from a single membrane composed of 40% soybean phosphatidylethanolamine, 40% soybean phosphatidylcholine, and 20% bovine phosphatidylserine. The aqueous phase, containing 100 mM NaCl, 5 mM tris (pH 7.2 initially), 2 mM $MgCl_2$, and 1 mM EDTA, was continuously stirred. The final concentration of TNF was 400 ng/ml.
31. Human U937 cells (American Type Culture Collection, ATCC) were washed four times in buffer A (100 mM choline chloride, 25 mM $MgCl_2$, 5 mM KCl, and 20 mM Hepes, with the addition of 7.17 mM NaOH to adjust the pH to 7.2). Each sample, containing 2×10^6 cells, was resuspended in 200 μl of buffer A after the last wash and equilibrated at 4°C for 15 min before the addition of 1 μg TNF (2 μl of a 0.5 mg/ml stock in 10 mM sodium phosphate and 0.2 M NaCl; pH 7) or 2 μl of buffer alone. Binding was allowed to proceed for 2 hours at 4°C. Then 10 μl of 20 mM ouabain in water or 10 μl of water alone were added, and the samples were incubated for 13 min at 37°C. Next, 10 μl of $^{22}NaCl$ [10 μM stock, 200 μCi/ml (Amersham, Arlington Heights, IL)] were added to each sample, and incubation at 37°C was continued for 10 min. Ice-cold PBS (0.8 ml; 10 mM sodium phosphate and 150 mM NaCl) was added to stop the flux of $^{22}Na^+$. The cells were pelleted in a microcentrifuge (Beckman) and washed twice with 1 ml of PBS. Aliquots (10 μl) of the first and last supernatants were removed for counting. Pelleted cells were solubilized by incubation for 15 min with 100 μl of 0.5% Triton X-100 in buffer A at 37°C. Solubilized cells and supernatant aliquots were mixed with 10 ml of liquid scintillant and counted at the ^{14}C setting of a Beckman scintillation counter. Na^+ uptake values are based on the presence of 9.365 mM Na^+ (radioactive plus cold). This assay is a modification of that of Smith and Rozengurt (*26*).
32. Fluorescence measurements were performed with a SPEX Fluorolog II spectrophotometer. In all studies, slit widths were 1.25 mm (band pass of 2.25 nm), the samples were stirred at 37°C, and the fluorescence measurements were taken in the ratio mode. The pH was adjusted by the addition of 1 N HCl or NaOH (<1% total volume) and monitored with a microelectrode. ANS (Eastman Kodak, Rochester, NY) binding studies were performed with 2 ml of buffer [0.15 M sodium citrate-phosphate-chloride, pH 8 initially (*27*)] having 4 μM of ANS and 10 μg of TNF. The graphs in Fig. 4, B and C, represent a single time scan with excitation and emission wavelengths of 380 nm and 480 nm, respectively. ANS was freshly prepared in the buffer. The quantum yield of fluorescence is dependent on environmental polarity (*17*) and is insensitive to pH over a wide range (*18*).
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Identification of a Major Growth Factor for AIDS–Kaposi's Sarcoma Cells as Oncostatin M

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Conditioned medium from human T cell leukemia virus type 2 (HTLV-II)–infected T cells supports the growth and long-term culture of cells derived from acquired immunodeficiency syndrome (AIDS)–associated Kaposi's sarcoma lesions (AIDS-KS cells). A protein of 30 kilodaltons was purified from conditioned medium that supports the growth of AIDS-KS cells. The amino-terminal sequence of this protein was identical to the amino-terminal sequence of Oncostatin M, a glycoprotein that inhibits the growth of a variety of cancer cells. Oncostatin M from conditioned medium stimulated a twofold increase in the growth of AIDS-KS cells at a concentration of less than 1 nanogram of the protein per milliliter of medium.

KAPOSI'S SARCOMA (KS) IS A MULTICENTRIC neoplasm found predominantly in males and frequently associated with human immunodeficiency virus (HIV) infection (*1, 2*). The KS lesion has a complex histology characterized by the proliferation of spindlelike cells with neovascularization. These lesions are often infiltrated by inflammatory cells, fibroblasts, and endothelial cells (*2, 3*). The mechanism underlying the pathogenesis of this complex tumor is not clearly understood.

Several cytokines, including interleukin-1, interleukin-6, and tumor necrosis factor–α, induce the growth of cells derived from AIDS-KS cells (*4–6*). These cells are morphologically similar to the spindlelike cells observed in KS lesions (*7*). The HIV-1 Tat protein has also been shown to enhance the growth of AIDS-KS cells at very low concentrations (*8*).

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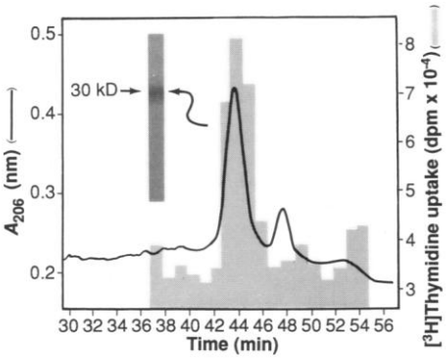

Fig. 1. A representative elution profile of the growth factor in reversed-phase HPLC on a C_{18} column (absorbance at 206 nm). A partially purified fraction from 38 CM with AIDS-KS cell growth activity was injected into an analytical μ Bondapak C_{18} column (Waters Instruments, Milford, Massachusetts) developed with a linear aqueous acetonitrile gradient (0 to 60%) containing 0.1% trifluoroacetic acid. The flow rate was maintained at 1 ml/min for 60 min. The growth activity of the fractions was measured with the AIDS-KS-3 cell line from a lung biopsy of an AIDS patient with lung KS (7). Cells were incubated in gelatinized 24-well plates (4×10^3 cells per well) with RPMI 1640 with 15% fetal bovine serum in the presence or absence of test fractions. The medium was replaced on day 3 and on day 4 was supplemented with 1.0 μCi of [^{3}H]thymidine per well. After overnight incubation, cell growth was determined by thymidine uptake over 24 hours. The results represent the average of assays done in duplicate. (**Inset**) SDS-PAGE of the purified factor. The fraction from HPLC with peak growth activity was separated by electrophoresis on a 15% polyacrylamide gel (*10*), and the protein was visualized with silver stain.

However, these factors did not support the long-term growth in culture of AIDS-KS cells (*4*). We have reported that continuous cultures of AIDS-KS cells could be maintained by means of conditioned medium from T cells immortalized by human retroviruses (HTLV-I and HTLV-II) (*4*). For this, conditioned medium from HTLV-II–infected T cells (38 cells) was particularly effective. Chronically activated normal $CD4^+$ T cells have many features in common with HTLV-II–infected and immortalized human T cells. In particular, others in our group have shown that all of these cells produce the activity that supports the culture of AIDS-KS cells (*9*). Earlier studies revealed that in 38-cell conditioned media (38 CM) the major active component for stimulating growth of AIDS-KS cells and for maintaining their long-term growth was a molecule of approximately 30 kD (*4*). We have now purified this factor from 38 CM.

The 38 CM used was concentrated by a Pellicon cassette system (Millipore, Bedford, Massachusetts) with a 10-kD cutoff limit. The concentrated material was then fractionated by ion-exchange chromatography on DEAE- and carboxymethyl Sepharose (Pharmacia, Piscataway, New Jersey). Various fractions obtained from these procedures were examined for the presence of AIDS-KS cell growth factor activity. The active fractions were further subjected to lectin affinity chromatography and metal chelate chromatography. The partially purified material was then subjected to repeated reversed-phase high-performance liquid chromatography (HPLC) on a preparative C_{18} column. The final step of purification was performed on an analytical C_{18} HPLC column with acetonitrile as the mobile phase.

Growth activity coincided with a single protein peak eluted from the HPLC column (Fig. 1). Fractions corresponding to the growth activity peak were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (*10*) and stained with silver. The only protein visualized in the fractions was a 30-kD polypeptide (Fig. 1, inset). Titration of growth activity present in the purified fraction revealed that the factor was capable of stimulating a twofold increase in the growth of AIDS-KS cells at a concentration of less than 1 ng of the protein per milliliter of medium (Fig. 2). A maximum 3.5-fold increase in [^{3}H]thymidine incorporation was obtained between 3 and 6 ng of the protein per milliliter of medium. Purified growth factor obtained from two preparations was analyzed separately for the NH_2-terminal amino acid sequence by Edman degradation in a gas-phase sequencer (Applied Biosystems, Foster City, California) (*11*). The amino acid sequence for the first 20 residues (*12*) was:

A-A-I-G-S-X-S-K-E-Y-R-V-L-L-G-Q-L-Q-X-Q-

A computer search for protein similarity revealed that this sequence is identical to the NH_2-terminal sequence of Oncostatin M. A peptide was synthesized corresponding to the NH_2-terminal sequence of Oncostatin M and was used to raise a rabbit monospecific antibody. This antibody recognized the purified 30-kD protein in both radioimmunoprecipitation (Fig. 3A) and immunoblot (Fig. 3B) assays.

The available literature on Oncostatin M is limited. The protein has been reported to be 30 kD in size and has been isolated from differentiated human histiocytic lymphoma cells (*13*) and from activated human T lymphocytes (*14*). Recombinant Oncostatin M expressed in mammalian cells (*15*) has inhibited the growth of a variety of cancer cells (*16*) and stimulated the growth of normal fibroblasts (*13, 16*). This cytokine has also been shown to effect morphological changes in some cells (*16*). The potent stimulating effect of Oncostatin M on the growth of AIDS-KS cells, which is very different from its inhibitory effect on other neoplastic cells (*16*), suggests that Oncostatin M may play a key role in the initiation of cellular events that lead to the development of KS lesions.

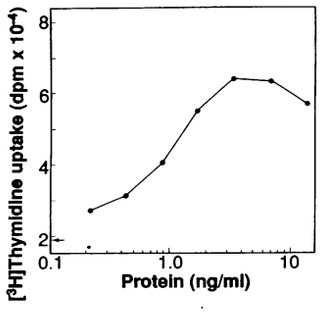

Fig. 2. Growth activity of the purified factor over 24 hours. An active fraction from HPLC was serially diluted and added to AIDS-KS-3 cells (7) for a growth activity assay as described under Fig. 1. The arrow indicates [^{3}H]thymidine uptake in the control medium without the factor.

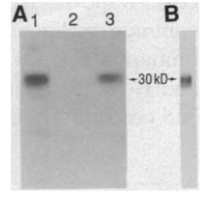

Fig. 3. Recognition of Oncostatin M by rabbit monospecific antibody against a synthetic peptide derived from the NH_2-terminal sequence of Oncostatin M. (**A**) Immunoprecipitation of ^{125}I-labeled Oncostatin M. The labeled protein was incubated overnight at 4°C with 20 μl of rabbit serum and 200 μl of a 10% suspension (v/v) of protein A Sepharose (Pharmacia, Piscataway, New Jersey) diluted to a final volume of 1 ml with phosphate-buffered saline (PBS)–TDS (10 mM sodium phosphate, pH 7.2, containing 0.5% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). The immune complexes were pelleted by centrifugation, washed three times with PBS-TDS, analyzed by SDS-PAGE (*10*), and visualized by autoradiography. In lane 1, a partially purified fraction (HPLC) was labeled with ^{125}I and run in parallel as a control. An identical sample was immunoprecipitated with the preimmune rabbit serum (lane 2) or a monospecific rabbit antibody to the Oncostatin M peptide (lane 3) (*10*). (**B**) Immunoblot analysis of the active fraction from HPLC purification (Fig. 1) using the rabbit monospecific antibody. An HPLC purified fraction (Fig. 1) was subjected to SDS-PAGE, and the protein was electrophoretically transferred to a nitrocellulose sheet. We blocked nonspecific binding sites by incubating the nitrocellulose sheet in blotto [5% nonfat milk and 0.01% Antifoam A (Sigma, St. Louis, Missouri)]. The nitrocellulose sheet was incubated overnight at 4°C with monospecific rabbit antiserum at a 1/100 dilution in blotto containing 4% normal goat serum. The sheet was washed with wash buffer [0.1 M NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100, and 20 mM sodium phosphate (pH 7.5)] and incubated with peroxidase-labeled goat antirabbit immunoglobulin G for 1 hour at room temperature. After washing the nitrocellulose sheet with repeated changes of wash buffer we treated it with diaminobenzidine-H_2O_2 to visualize the protein band.

Our working concept is that one or more factors released by retrovirus-infected T cells are critical in the development of KS because they initiate cellular events that lead to the production of cytokines. These cytokines, in turn, have autocrine and paracrine effects and thus produce histological changes characteristic of AIDS-KS (*17*). Immunological and molecular probes for Oncostatin M will be valuable tools to study its role in KS development.

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18. We thank Y. Kim for expert assistance in the amino acid sequence analysis. Supported in part by the National Cancer Institute, Department of Health and Human Services, under contracts NO1-CP-73723 and NO1-CO-74101 with Advanced BioScience Laboratories, Inc.

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Oncostatin M as a Potent Mitogen for AIDS-Kaposi's Sarcoma–Derived Cells

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Oncostatin M, a cytokine produced by activated lymphoid cells, regulates the growth and differentiation of a number of tumor and normal cells. In contrast to its effects on normal endothelial and aortic smooth muscle cell cultures, Oncostatin M was a potent mitogen for cells derived from acquired immunodeficiency syndrome–related Kaposi's sarcoma (AIDS-KS). After exposure to Oncostatin M, AIDS-KS cells assumed a spindle morphology, had an increased ability to proliferate in soft agar, and secreted increased amounts of interleukin-6. Oncostatin M RNA and immunoreactive Oncostatin M protein were found in AIDS-KS–derived cell isolates. These results suggest that Oncostatin M may play a role in the pathogenesis of AIDS-KS.

AIDS-RELATED KAPOSI'S SARCOMA (AIDS-KS) is a malignant, neoplastic proliferation of mesenchymal cells. Kaposi's sarcoma (KS) is the most common malignancy complicating human immunodeficiency virus (HIV) infection and is the source of significant morbidity and occasional mortality (*1*). Although effective therapy exists for mild to moderate cases of KS (*2*), many patients develop aggressive cases of the disease that complicate treatment of both the underlying HIV infection and the associated opportunistic infections (*3*). New therapeutic modalities are needed to more effectively treat AIDS-KS.

The introduction of routine cell culture of AIDS-KS–derived spindle cells (AIDS-KS cells) (*4*) has made possible the in vitro study of cytokines that may be involved in the pathogenesis and regulation of proliferation of AIDS-KS cells. By means of these culture systems, interleukin-6 (IL-6) (*5*) and the transactivating protein of HIV, Tat (*4, 6*), were identified as important growth factors for AIDS-KS–derived cells. A variety of other cytokines, including interleukin-1β (IL-1β), tumor necrosis factor–α (TNF-α), gamma-interferon, transforming growth factor–β (TGF-β), platelet factor 4, and interleukin-4 (IL-4), have been shown to modulate IL-6 and to have effects on the proliferative rate of cultured AIDS-KS cells (*6, 7*). Alteration in the levels of one or more of these cytokines has been demonstrated in patients with various stages of HIV infection (*8*), and the potential role of these cytokines in the pathogenesis and proliferation and progression of KS has been discussed (*8*).

Oncostatin M is a 28,000- to 36,000-kD polypeptide cytokine produced by activated T lymphocytes and phorbol 12-myristate 13-acetate (PMA)–treated monocytic cell lines (*9–11*). Although originally identified by its ability to inhibit the growth of A375 melanoma cell lines (*10*), Oncostatin M has a wide variety of in vitro activities including growth regulation of malignant and normal cell lines (*9, 11*). Oncostatin M also increases the expression of IL-6 in cultured human endothelial cells (*12*). Because of the effects of Oncostatin M on these cells, its production by both activated T lymphocytes and PMA-treated U937 cells, and the belief that AIDS-KS–derived cells are mesenchymal-derived cells, we investigated the effects of Oncostatin M on AIDS-KS–derived cell lines.

Recombinant Oncostatin M at low concentrations was found to be one of the most potent mitogens for AIDS-KS–derived cell lines (Fig. 1). For example, for the mitogenesis of AIDS-KS cell lines the half-maximal concentration of Oncostatin M was ~2 ng/ml of medium (70 pM) in short-term culture. Long-term cultures of AIDS-KS cells show that the optimal concentration for proliferation is approximately 250 pg/ml (*6*). These results are less than the half-maximal concentration for mitogenesis of approximately 20 ng/ml for IL-1β and TNF-α. Similar to the effects of Oncostatin M on endothelial cells, mitogenesis of KS

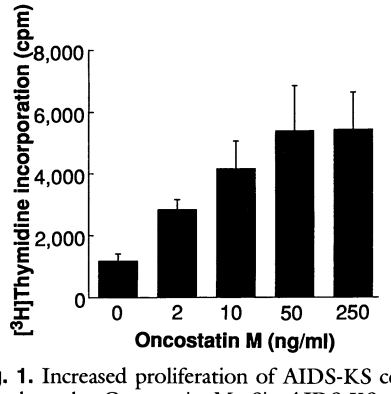

Fig. 1. Increased proliferation of AIDS-KS cells in culture by Oncostatin M. Six AIDS-KS cell lines (KSL1, KSL2, KSL4, KSL5, KSL6, KSL7) were exposed to recombinant Oncostatin M at concentrations from 1 to 1500 ng/ml in serum-free media; representative data from one cell line (KSL4) performed in quadruplicate are presented (mean ± SD). Proliferation was measured by [^{3}H]thymidine incorporation over 18 hours, and proliferation assays were performed as described (*5*).

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