

CORRELATION BETWEEN AIDS-RELATED KAPOSI SARCOMA HISTOLOGICAL GRADE AND IN VITRO BEHAVIOR: REDUCED EXOGENOUS GROWTH FACTOR REQUIREMENTS FOR ISOLATES FROM HIGH GRADE LESIONS

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ABSTRACT

Kaposi sarcoma, the most common AIDS-associated malignancy, affects 10-30% of all AIDS patients. To date, research into the biological characteristics of AIDS-related Kaposi sarcoma (AIDS-KS) derived cell lines has been based on cultures established from skin explants or pleural effusions/peritoneal fluids. We have established several AIDS-KS lines from biopsy confirmed oral mucosal and epidermal AIDS-KS lesions and have found a correlation between AIDS-KS lesional grade and in vitro cellular growth characteristics. In comparison to epidermal AIDS-KS lesions, mucosal AIDS-KS lesions frequently possessed both a more advanced histologic grade and demonstrated a greater capacity to proliferate in minimal medium. We report the ability of AIDS-KS isolates from high grade lesions to sustain proliferation (greater than 60 population doubling levels) in medium not supplemented with endothelial cell growth supplement and/or cytokine rich conditioned medium. These findings indicate that AIDS-KS cells isolated from high grade lesions have reduced requirements for exogenously provided growth supplements, and suggest that increased autologous cytokine production accompanies AIDS-KS lesional progression.

Prior to the Acquired Immunodeficiency Syndrome (AIDS), epidemic Kaposi sarcoma (KS) was rare in the United States, and was confined primarily to elderly men of Mediterranean descent, men from Equatorial Africa, and transplant recipients (1,2). However, AIDS-related Kaposi sarcoma is now the most common AIDS associated malignancy, with an overall incidence of 10-30% (3). Currently, AIDS-KS is diagnosed in the latter stages of AIDS, with a mean patient survival time following diagnosis of 18-24 months (3,4). Although AIDS-KS affects both the mucous membranes and skin, extensive mucosal disease (nodular, diffuse) is associated with a poorer overall prognosis (5-7).

Studies of AIDS-KS pathobiology became feasible only after the development of *in vitro* culture techniques applicable for AIDS-KS cells (8). The initial laboratories to establish AIDS-KS cells employed specific *in vitro* growth conditions which included: culture in a base medium (e.g. Iscoves DMEM, RPMI 1640, or M-199), supplemented with 10-20% fetal bovine serum, endothelial cell growth supplement, and a cytokine rich conditioned medium from human retrovirus infected cells, usually HTLV-II (8-11). The primary focus of these initial studies was the determination of the histogenesis of the AIDS-KS cell, and its

associated oncogenic potential (8-11). These original studies relied on two primary sources of AIDS-KS tissue: (which were frequently not microscopically confirmed as KS) skin explants (12,13-15) and pleural/peritoneal effusions (8-10,16).

The objective of our study was to investigate whether there was a correlation between lesional histological grade and cellular capacity to sustain proliferation during culture with reduced exogenously supplied growth supplements. We determined that relative to AIDS-KS cells isolated from low histological grade lesions, AIDS-KS isolates from high grade lesions demonstrated

accelerated growth kinetics and enhanced proliferative capacities during culture with minimal supplemental growth factors. Our findings imply that inherent, histological grade dependent differences exist in the growth modulation of AIDS-KS cells.

MATERIALS AND METHODS

Histologic Confirmation of AIDS-KS

Prior to excisional biopsy of the suspected AIDS-KS lesion, an examination was conducted to determine the extent and clinical presentation of the patient's lesion(s). A

TABLE 1
Various Experimental Media Compositions

MEDIUM COMPONENT	1	2	3	4	5	6	7	8	9	10	11	12	13
M-199, 15 mM HEPES, 1.1 µg/ml Na Pyruvate, 2.8 µg/ml L-glutamine	+	+	+	+	+	+	+	+	+	+	+	+	+
20 % FBS					+	+							
15 % FBS	+	+	+	+			+						
8.5% FBS								+					
2.0% FBS									+	+	+	+	
5% HS (Human Serum)	+	+	+	+									
ECGS (120 µg/ml)	+	+		+	+					+		+	
HEPARIN (90 µg/ml)	+	+	+										
Mo-T (HTLV-II condition-medium)	+										+	+	+
Mean Medium Stimulatory Index ¹	99.0	93.6	44.9	52.6	31.0	34.1	29.6	27.2	13.3	14.3	17.0	27.4	7.5

¹ Stimulatory index based on cell density of cultures on day 10 in appropriate medium as compared to density in M199 base

portion of each biopsy was submitted for light microscopic examination; 22 out of 25 biopsies were microscopically confirmed by two Board Certified Oral Pathologists as AIDS-KS. Twenty AIDS-KS cultures (obtained from both oral mucosal and epidermal lesions) were isolated.

Isolation of AIDS-KS Cell Lines

AIDS-KS cell lines were isolated as described (17). Briefly, biopsy material was digested with 2 mg/ml of types Ia, II, and IV collagenases in 20 ml of 0.01% trypsin (collagenase type II and trypsin Worthington Biochemical, Freehold, N.J., and types Ia and IV Sigma Chemical St. Louis, MO) for 60-120 minutes. Cultures were initiated in medium 1 (*Table 1*) consisting of: M-199 (Gibco, Grand Island, NY), 15 mM HEPES, 2.9 µg/ml l-glutamine, 1.1 µg/ml Na pyruvate, 90 µg/ml sodium heparin (Sigma), 150 µg/ml endothelial cell growth supplement (ECGS, prepared in-house from bovine brain), 15% fetal bovine serum (FBS, Hyclone, Logan, UT), 5% male human serum, 20% Mo-T (an HTLV-II infected T-lymphocyte cell line) conditioned medium, 4 µg/ml amphotericin B, 50 µg/ml amikacin sulfate, and 25 µg/ml imipenem-cilastin sodium. Cells were plated on human fibronectin that contained 5% Mo-T conditioned medium. Once the primary cultures became confluent and were determined to be free of microbial contamination, the antibiotics were removed from the medium. At passage three, sister flasks were split (harvested by trypsin, and transferred 1 flask to 2 flasks), and placed into either medium 1, or medium 2. Medium 2 (*Table 1*) consisted of the same base medium (M-199), supplements, and serum as medium 1, but did not contain any Mo-T conditioned medium in either the medium or the human fibronectin.

Additional Cell Lines

Mo-T, an HTLV-II transformed cell line (23), and normal human palatal mesenchymal

cells (24) were obtained from the American Type Tissue Culture Collection and maintained in their recommended medium (18).

Growth Curves

Baseline growth curves were determined for each cell line at passage 6 by growing the cultures to 80% confluency in their respective normal growth medium (medium 1 or 2), washing the cells with phosphate buffered saline (PBS), and culturing for an additional 48 hours in M-199 "base-medium" (M-199 with 15 mM HEPES, 2.9 µg/ml L-glutamine, and 1.1 µg/ml sodium pyruvate) to remove any residual serum/medium stimulatory effects. The cells were trypsinized and seeded at a density of 50,000 cells per well in human fibronectin coated 6 well plates with 4 ml/well of either medium 1 or 2. At each time point triplicate wells were harvested and quadruplicate cell counts made with a Coulter counter. An additional 0.5 ml of fresh medium was added to each well on days 2, 4, 6, 8.

Media Formulation Experiments

Thirteen different formulations of medium were tested for their ability to support the short term growth of the cell lines. The composition of the media were as described in *Table 1*.

All growth medium formulation experiments were conducted on passage 6 cells grown to 80% confluency in their respective normal growth medium (medium 1 or 2). The cells were then washed with PBS, and cultured for 48 hours in M-199 "base-medium" to remove any residual medium supplements/serum stimulatory effects. Cells were trypsinized, and seeded in human fibronectin coated 24 well plates at 10,000 cells/well in duplicate or triplicate wells in 1 ml/well of the respective growth medium. Plates were harvested on days 1, 3, 5, 7, 10 and quadruplicate counts made of each well with a Coulter counter. Additional 0.5 ml of the respective test medium was added to each well on days 3, 5 and 7. Stimulatory indices were

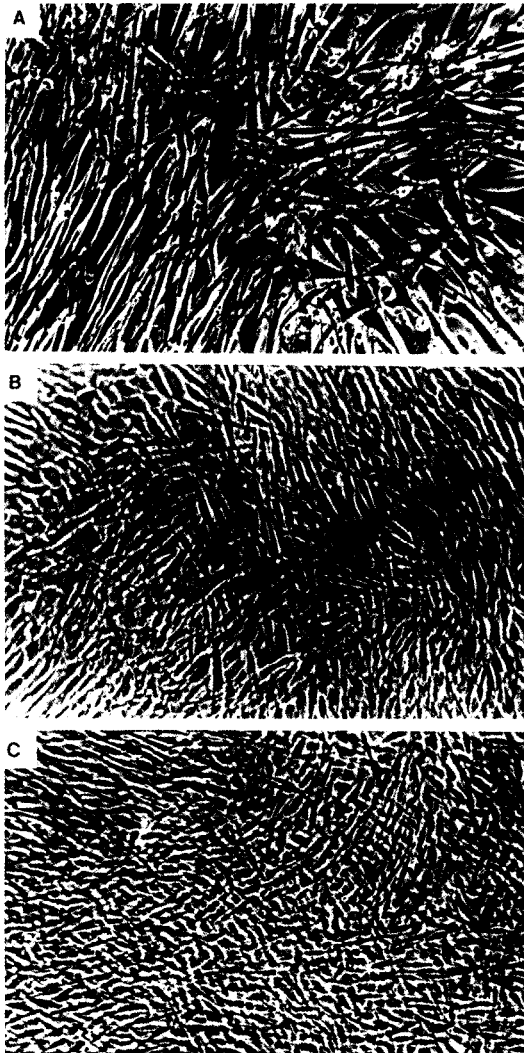


Fig. 1. Representative photomicrographs, taken at 50X (image scale) of human cells during culture in complete medium supplemented with conditioned medium from Mo-T. AIDS-KS isolates from a low histological grade lesion are characterized by a cellular heterogeneity, and a lack of contact inhibition (A). In contrast, mucosal derived AIDS-KS cells from a high histological grade lesion (B) display a more homogeneous appearance, along with a higher cell density and bear a marked similarity to human embryonic mesenchymal cells (C).

determined for each cell line and medium combination by comparison of the cell densities at harvest in the test medium vs the M-199 “base-medium.”

Long Term Growth of Cell Lines Under Reduced Serum Concentrations

Cells at passage 6 were grown to 80% confluency in either medium 1 or 2, then rinsed with PBS and switched to M-199 “base-medium” for 48 hours. The cells were then trypsinized, and transferred to human fibronectin coated 6 well plates at a concentration of 50,000 cells/well. Cells were grown in duplicate wells in M-199 “base-medium” supplemented with 15%, 8.5% or 2% FBS (media 7, 8, 9). One ml of the appropriate fresh medium was added to each well every third day. Upon confluence, the cells were grown in medium 7 or 8 and split 1:4, and those grown in medium 9 were split 1:3, and transferred to new, human fibronectin coated, 6 well plates.

RESULTS

Specific, stringent criteria were employed to document that our cultures were AIDS-KS in origin. First, a portion of all AIDS-KS tissue biopsies was submitted for light microscopic examination to confirm the diagnosis of AIDS-KS. Second, our AIDS-KS cell lines, during growth both in medium 1 and 2 conditions, showed characteristics consistent with a transformed phenotype—a lack of contact inhibition, loss of anchorage dependence, and a concurrent increase in intercellular adhesion (17). The AIDS-KS lines isolated in our laboratory also possessed a spindle cell morphology (*Fig. 1*), a recognized phenotypic feature of cultured AIDS-KS cells (1,10,11). Finally, our AIDS-KS cell lines demonstrated high autologous production of interleukin 6, a characteristic feature of AIDS-KS cells (10,15).

Light microscopic examination of the AIDS-KS biopsy tissues (corresponding to the tissues used for culture) showed that mucosal lesions of AIDS-KS frequently had a more advanced histologic grade than the epidermal AIDS-KS lesions (*Fig. 2*). The higher grade AIDS-KS specimens displayed numerous

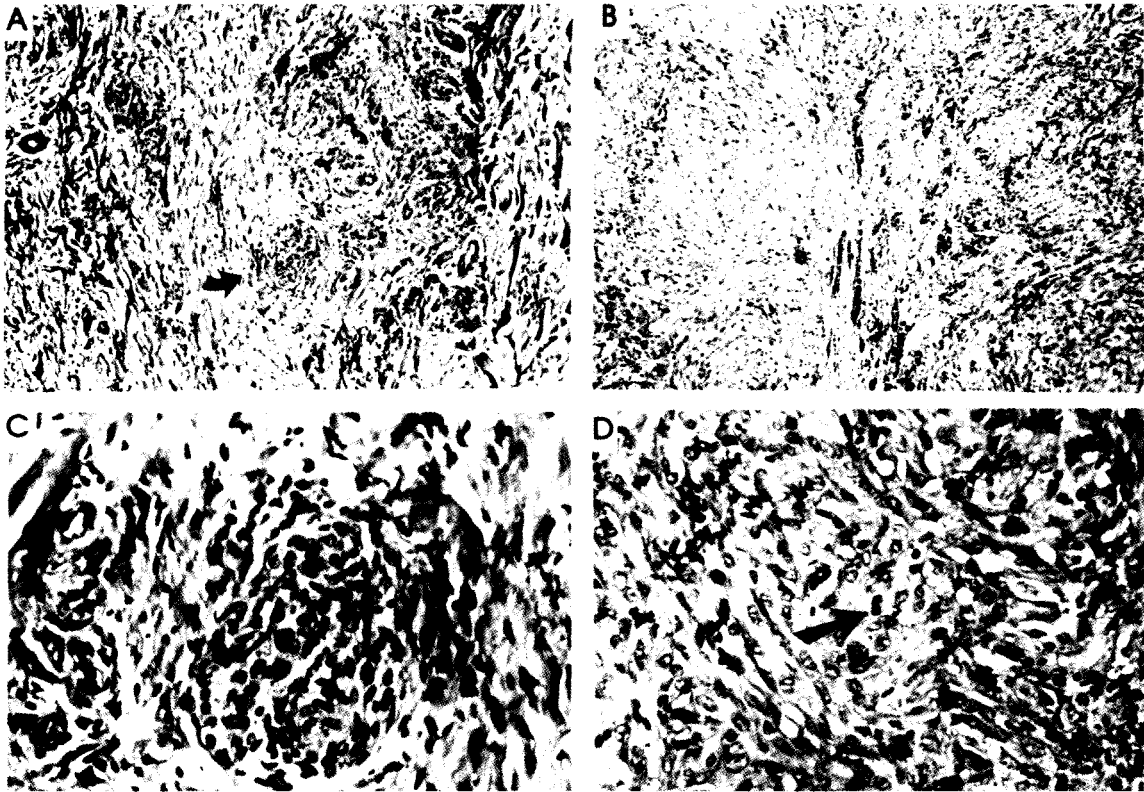


Fig. 2. Representative photomicrographs of paraffin embedded, hematoxylin and eosin stained AIDS-KS biopsies. AIDS-KS epidermal lesions (A 25.2X, C 100X, image scale) frequently possessed a low histological grade appearance, characterized by focal, mitotically inactive AIDS-KS lesional aggregates (arrow in A) that resemble granulation tissue. In contrast, AIDS-KS mucosal isolates from high grade lesions (B 25.2X, D 100X, image scale) demonstrated a high density of AIDS-KS lesional cells in conjunction with marked cellular pleomorphism and obvious mitotic activity (arrow in D).

mitotically active AIDS-KS lesional spindle cells in addition to frequent slit shaped, highly permeable vascular channels. In contrast, the epidermal AIDS-KS lesions often resembled exuberant granulation tissue (consistent with early, and/or low grade lesions of AIDS-KS), and contained sparse aggregates of the lesional AIDS-KS spindle cells interspersed in subacutely inflamed fibrovascular connective tissue.

Phenotypic differences, which were associated with the lesional histological grade, were apparent in the cultured AIDS-KS cells. Whereas the KS cells isolated from the lower grade epidermal lesions showed a greater overall cellular heterogeneity, and possessed

an elongated, spindled phenotype suggestive of myoblasts (*Fig. 1*), the high grade mucosal isolates were more homogeneous and possessed a morphology reminiscent of embryonic palatal mesenchymal cells (*Fig. 1*). Although our immunohistochemistry results showed infrequent cellular positivity for smooth muscle alpha actin, desmin, and Factor VIII, only vimentin was uniformly positive in each of the AIDS-KS lines. This finding is consistent with the accepted concept that the AIDS-KS cell is derived from a primitive, pluripotent, mesenchymal stem cell (1,13,19), and is in agreement with the AIDS-KS immunohistochemistry results reported by other investigators (11,20,21).

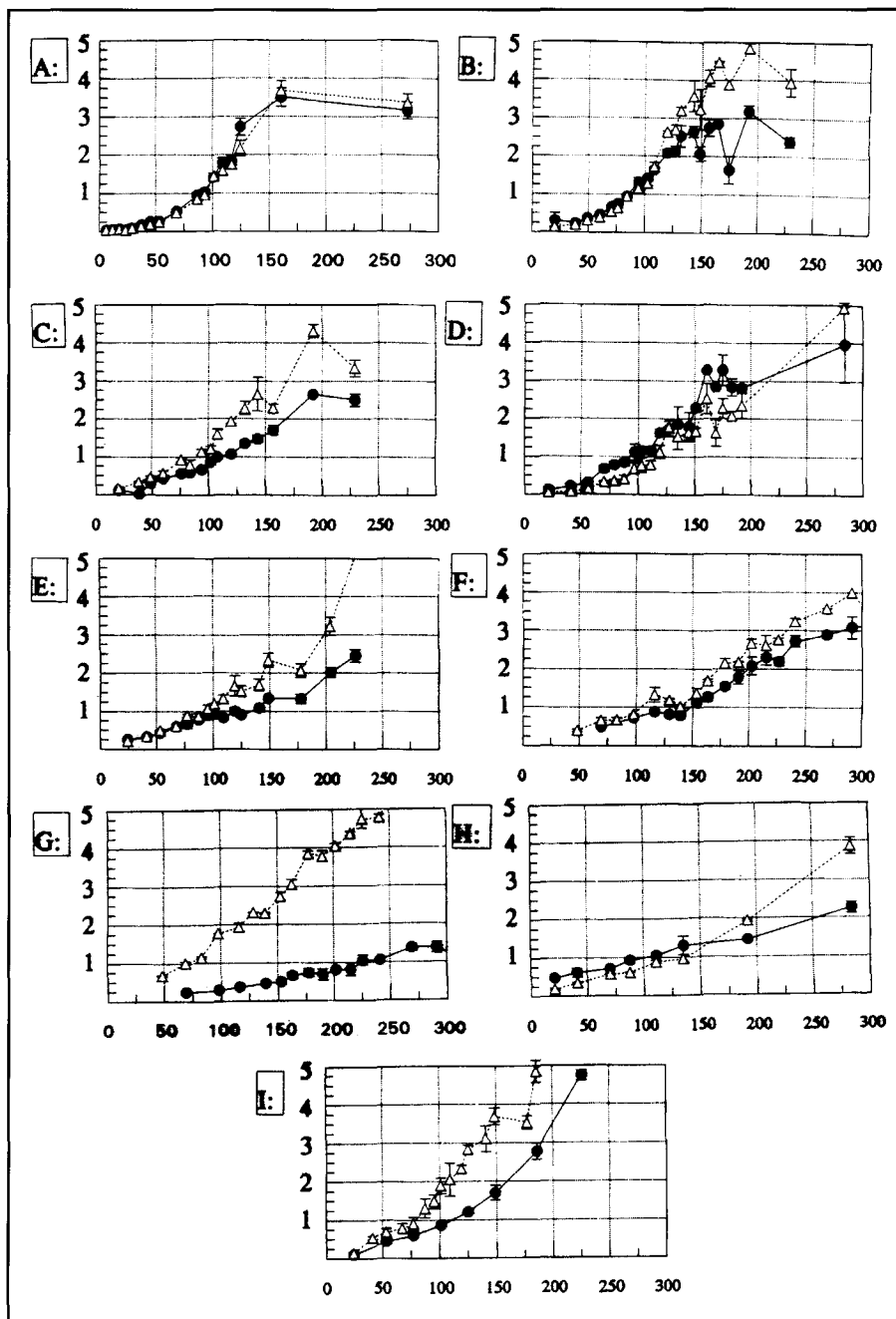


Fig. 3. Growth curves of AIDS-KS cell lines in medium 1 (open triangles) and medium 2 (closed circles). Plot generated with ordinate axis representing total cells number ($\times 10^6$) and abscissa axis represents hours in culture. Cultures were initiated at 50,000 cells/well in 6 well plates. Each datum point represents the mean value of quadruplicate counts (\pm S.D.) on a coulter counter of triplicate wells. A: Mucosal (high grade) derived cell line 93-2141A. B: Mucosal (high grade) derived cell line 93-2141B. C: Mucosal (high grade) derived cell line 92-3239. D: Mucosal (high grade) cell line 94-0683. E: Mucosal (high grade) derived cell line 93-3156. F: Epidermal (low grade) cell line KS-93-0897. G: Epidermal (low grade) cell line 92-1930. H: Epidermal (low grade) cell line 94-0529. I: Epidermal (low grade) cell line 92-2673.

TABLE 2
Cell Strain Doubling Times

TISSUE ORIGIN	CELL LINE	DOUBLING TIMES IN HOURS	
		MEDIUM 1	MEDIUM 2
ORAL MUCOSA (High Histological grade)	93-2141A	35.3	38.8
	93-2141B	45.6	83.4
	92-3239	49.2	65.6
	94-0683	36.5	50.2
	93-3156	61.3	103.4
	MEAN	45.6	68.3
EPIDERMAL (Low Histological grade)	92-1930	59.0	81.8
	93-897	72.0	70.0
	94-0529	81.4	163.5
	93-2673	73.7	56.0
	MEAN	71.5	92.8

Studies conducted to determine the *in vitro* growth potential of the AIDS-KS cells revealed that regardless of the histological grade (low or high), the AIDS-KS cells uniformly proliferated in both medium 1 or 2 (Table 2, Fig. 3), with shorter doubling times noted for all cultures during growth in the cytokine-rich medium 1. However, there were also histological grade associated differences in the AIDS-KS cellular growth capacities. Whereas the average doubling times for AIDS-KS cells isolated from high grade lesions was 45.6 hours and 68.3 in medium 1 and medium 2, respectively, the AIDS-KS lines isolated from low grade lesions averaged 92.8 hours (medium 2) and 71.5 hours (medium 1) to complete a population doubling. These differences in proliferative rates between cells isolated from high and low grade AIDS-KS lesions during culture in medium 1 were statistically significant ($p=0.03$, Mann Whitney U, two tailed test). Relative to the AIDS-KS cultures derived from low grade lesions, the high grade isolates

proliferated more rapidly during culture in medium 2; however, these differences were not statistically significant.

Additional studies were conducted to evaluate the growth stimulatory potential of specific medium components. These results revealed that regardless of histological grade all of the AIDS-KS cell lines proliferated (for at least 2 population doubling levels) in each of the test media (Table 1, Fig. 4). Further, proliferative capacity was proportionate to the degree of enrichment of the medium. Based on the stimulatory indices, the media evaluated can be ranked from the most to the least supportive of cell proliferation (Fig. 4).

Our media component/stimulatory indices studies demonstrated, as anticipated, that the serum concentration was a primary determinant in the regulation of cellular proliferation. Previously, we determined that during culture in the cytokine rich medium 1, both low grade and high grade AIDS-KS isolates sustained multiple (≥ 30) population doublings (data not shown). To evaluate the

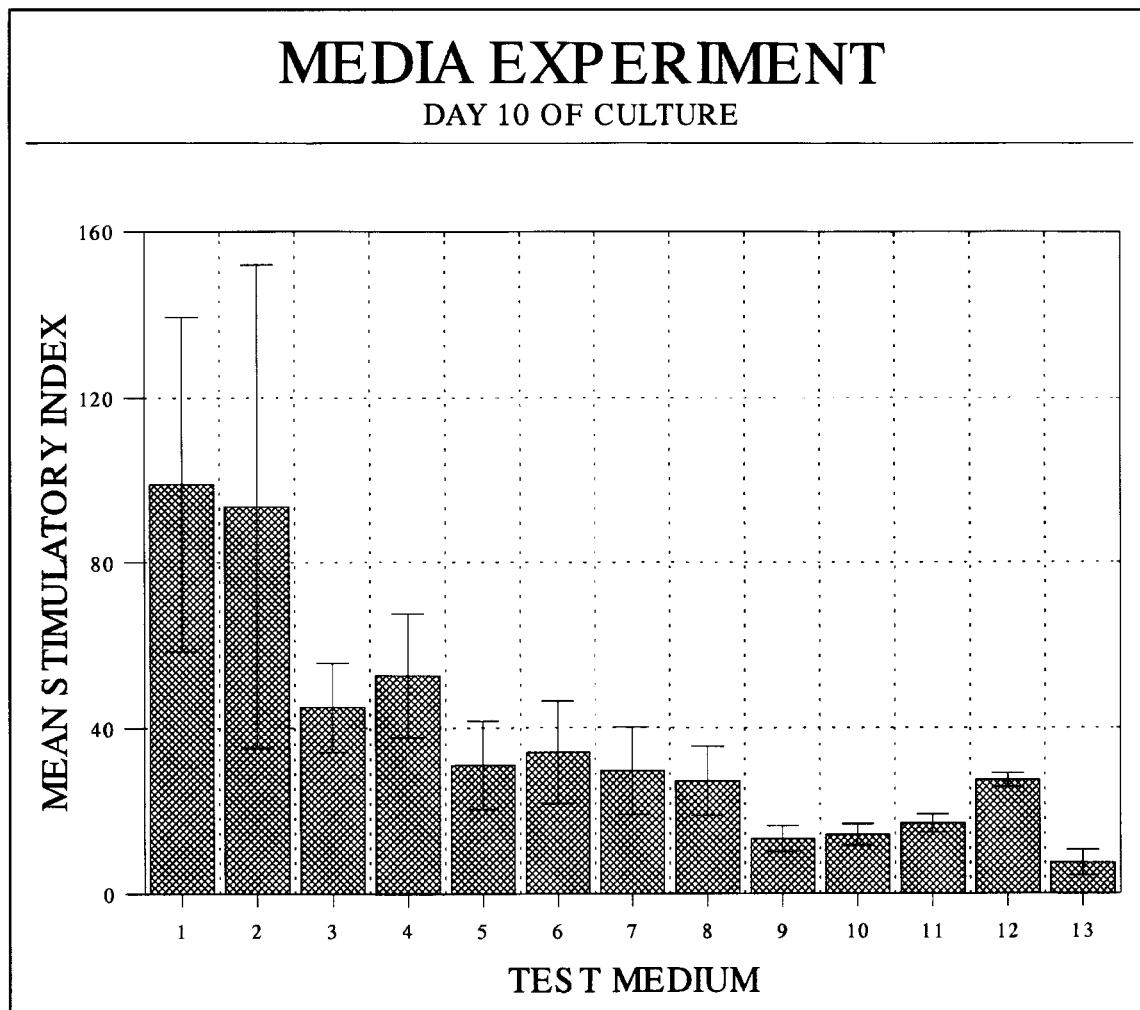


Fig. 4. Stimulatory indices of test media. Plot represents the mean stimulatory index (\pm S.D.) of each growth medium on day 10 as compared to growth in M199 base-medium. Cultures are serum starved for 48 hours prior to seeding at 10,000 cells/well in duplicate wells. Cell numbers are quantitated by calculation of the mean cell count based on quadruplicate counts with a Coulter counter of each well. Medium 1-13 compositions as per Table 1.

cellular dependency on FBS, as well as to investigate the AIDS-KS cellular capacity to proliferate independently of exogenously supplied Mo-T conditioned medium or ECGS, a series of experiments were conducted. Specifically, these experiments evaluated the capacity of AIDS-KS cells to sustain proliferation during multiple passages in M-199 "base-medium" supplemented with either 15%, 8.5%, or 2% FBS (media 7, 8, 9). Pronounced, AIDS-KS histological grade

dependent differences were noted compared with the AIDS-KS cellular capacities to sustain proliferation in "reduced growth factor" medium (reduced serum, no Mo-T conditioned medium or ECGS) (Fig. 5). AIDS-KS cells isolated from low histological grade lesions became senescent after four population doubling levels in each of the test medium (media 7,8,9). In contrast, AIDS-KS isolates obtained from high histological grade lesions retained their growth potential (≥ 60

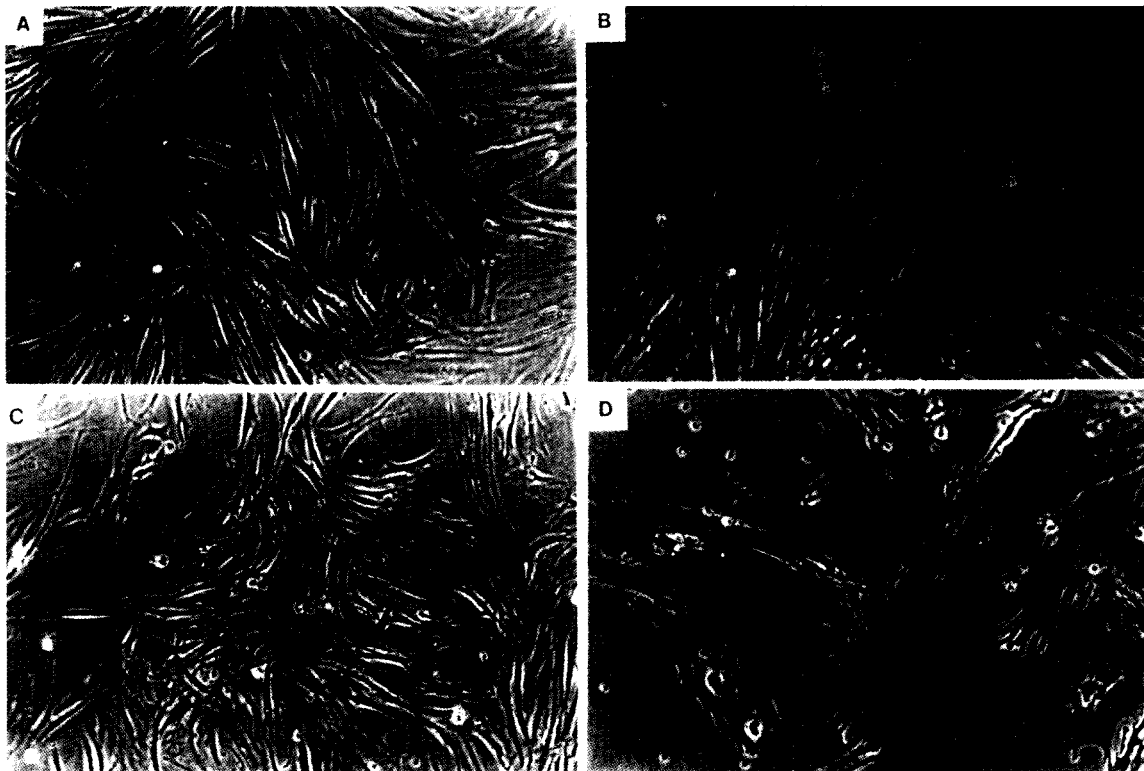


Fig. 5. Effects of reduced serum on the morphology of AIDS-KS derived cell lines. Cells are maintained in duplicate wells of 6 well plates in the respective medium and split at either a 1:3 ratio (2% FBS) or a 1:4 ratio (15% or 8.5% FBS) upon reaching confluency. Mucosal (high grade) derived cell lines 94-0683 in 15% FBS at passage 5 in: 15% FBS (A), 8.5% FBS (B), 2% FBS (C). Note the decreased cell density and increased cell size, inclusive of elongated ends, in 2% FBS. D: Epidermal (low grade) AIDS-KS cell line 93-0897 in 8.5% FBS at passage 2. Cellular autolysis is evident, and was noted in all low histological AIDS-KS lines cultured in reduced serum at passage 2.

population doublings) in all of the tested media. However, when cultured in the 2% FBS medium (medium 9), the AIDS-KS cells obtained from the high grade lesions demonstrated cellular perturbations as indicated by an overall increase in cell size, appearance of stress fibers, and a lower final cellular density at culture confluence (Fig. 5).

DISCUSSION

The findings presented in this paper have demonstrated, for the first time, a correlation between the *in vivo* AIDS-KS lesional grade and *in vitro* cellular growth characteristics. Specifically, AIDS-KS strains isolated from

high histological grade lesions have a greater capacity to proliferate *in vitro* as demonstrated by more rapid population doubling times and retention of growth potential in minimal medium. Our data suggest that either increased autologous growth factor production, or decreased growth factor requirements by the high grade AIDS-KS isolates account for their greater *in vitro* growth potential.

Light microscopic evaluation of the AIDS-KS biopsies showed that the majority of the high grade lesions were oral mucosal in origin. Two possible explanations may account for these findings. Intramucosal lesions, because of their location, may be less conspicuous to

the patient, and therefore not detected until later in the clinical course. On the other hand, many of the mucosal AIDS-KS lesions were biopsied during the patch/plaque clinical stages, and still demonstrated an advanced histologic grade. A second consideration is that mucosa may be a target site for AIDS-KS growth promoting cytokines, growth factors and hormones, which may act synergistically to accelerate AIDS-KS lesional progression from hyperplasia to neoplasia.

Both inter-cell line and intra-cell line variations in AIDS-KS cellular morphology were apparent; with the low grade AIDS-KS isolates showing the most intraline morphological heterogeneity. Although these findings are consistent with the concept that AIDS-KS is polyclonal in nature (1,5), the greater degree of homogeneity noted in the high grade AIDS-KS cultures suggests that the high grade AIDS-KS lesions are progressing toward a monoclonal cellular proliferation. The doubling times that we report compare favorably with the ranges of doubling times that have been described by others for AIDS-KS cell lines in HTLV-II infected lymphocyte conditioned medium (9), or at early passages in M-199 supplemented with 10% human serum (11), or in 5% FBS supplemented DMEM (13). We determined that AIDS-KS lines isolated from high grade lesions possessed shorter population doubling times relative to AIDS-KS cells isolated from low histological grade lesions during culture in either medium 2 or medium 1, and found statistically significant differences during culture in medium 1. Whereas medium 1 contains multiple cytokines (IL-6, Oncostatin M, PDGF, GM-CSF, TNF- β) known to stimulate AIDS-KS cells; medium 2, although less cytokine rich, still contains abundant growth factors with the inclusion of ECGS and 20% sera (15% FBS, 5% human serum). It is well established that the presence of an exogenous cytokine can not only function to upregulate autologous cytokine production (22), but can also stimulate autologous production of related cytokines, thereby

triggering a cytokine cascade (23). Therefore, the enhanced responsiveness of the high grade AIDS-KS isolates to the exogenous cytokines suggests that advanced histologic grade AIDS-KS cells increased cytokine receptor expression and/or an enhanced ability to stimulate autocrine production of growth promoting cytokines (22-24).

The results from our media stimulatory indices studies followed predictable concentration/dose effects, with more enriched medium resulting in more rapid growth of the cells. Notably, medium supplemented with Mo-T resulted in a higher stimulatory index than did the addition of ECGS (a crude extract that contains both acidic FGF and basic FGF). This difference can be attributed to the variety and higher concentrations of cytokines present in the Mo-T conditioned medium. As would be anticipated, the FBS concentration was the one medium component that had the greatest influence on the rate of cell proliferation. Due to the admixture of components in FBS, it is unlikely that one element alone is exclusively responsible for stimulating cell proliferation. The large standard deviations detected during the determination of the medium stimulatory indices most likely reflect the extent of AIDS-KS cellular heterogeneity, at both the interline and intraline levels.

We determined that AIDS-KS cells isolated from low histological grade lesions were unable to proliferate for more than 2 passages (8 population doublings) in reduced serum that contained no Mo-T conditioned medium. In contrast, the high grade AIDS-KS isolates survived in excess of 60 population doublings in M-199 "base-medium" supplemented with only 2% FBS (medium 9). These results demonstrate that not only are there distinct, histological grade dependent differences in AIDS-KS cellular proliferation potential, but also challenge the established concept that for long term culture AIDS-KS lines require medium that is supplemented with high concentrations of serum and cytokines/growth factors (8-10,12-14,16). This

ability of advanced histological grade AIDS-KS cells to proliferate (≥ 60 population doublings in medium 9) under "reduced growth factor" conditions suggests, that relative to low histological grade isolates, these cells have either diminished growth factor requirements, or are better autologous producers of growth potentiating cytokines. Currently, studies are ongoing in our laboratory to investigate whether there are histological grade dependent differences in AIDS-KS cellular autologous cytokine production during both the proliferative growth and environmentally stressed states.

This work is the first to include the contribution of the *in vivo* lesional histological grade with the *in vitro* evaluation of AIDS-KS cellular growth characteristics. Our results demonstrate a correlation between the lesional histological grade and *in vitro* growth potential, and show that the higher grade, oral-mucosal AIDS-KS cell lines retained proliferative capacity during culture in minimal medium. These findings suggest that increased autologous growth factor production, and/or reduced exogenous growth factor requirements, account for the increase in proliferative potential and biological aggressiveness that accompanies AIDS-KS lesional progression.

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