ENDOTHELIAL TRANSDIFFERENTIATED PHENOTYPE AND CELL-CYCLE KINETICS OF AIDS-ASSOCIATED KAPOSI SARCOMA CELLS

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ABSTRACT

The nature of Kaposi sarcoma (KS) (vascular malignancy vs. discordant angiogenesis) and lineage of the progenitor cell remain unclear. Therefore, AIDS-KS enzyme isolate cultures were prepared from excised skin lesions. Endothelial marker positivity for Factor VIII related antigen (F8RAg), Ulex europaeus agglutinin (UEA), and angiotensin-converting enzyme (ACE) were determined by fluorescence microscopy (FM) and flow cytometry (FCM). DNA cell-cycle analysis was performed using FCM. KS lesions showed large thick-walled channels (F8RAg and UEA strongly +), narrow vascular slits and thin-walled lakes (F8RAg and UEA weakly +), and non-prominent spindle cells (F8RAg and UEA almost uniformly negative). KS cultures yielded heterogenous populations of spindle, stellate, and flattened endothelial-like cells, displaying positivity for F8RAg (64±3%; mean±SE), UEA (40±9%), and ACE (81±9%). When injected subcutaneously in the nude mouse these cells failed to produce tumors. During contact inhibition induced quiescence, KS cultures exhibited a high G_2M (18±3%) compared to non-KS (7±4%; p<0.04), evidence of an altered proliferative potential consistent with a transdifferentiated or transformed phenotype.

Although vascular and specifically lymphatic endothelium has been favored as the cell of origin for the "spindle cell" in Kaposi sarcoma (KS) (1-3), recent reports describing the morphology and immuno-histochemical staining patterns of cells derived from long-term culture of AIDS-KS lesions and fluids have called into question an endothelial vascular origin of KS (4,5). In light of the well-recognized phenotypic diversity of endothelial cells derived from different sites or grown under different culture conditions (6), these recent conclusions warrant reexamination. To characterize more fully cells derived from biopsied KS skin lesions in HIV-seropositive patients with AIDS, we used previously established techniques for successful isolation of lymphatic and benign neoplastic endothelium in long-term culture (7). We compared the phenotypic characteristics of these cells to cells populating the reference KS lesions. Focusing in large part on flow cytometric methods for quantitation, we determined percent endothelial cell marker positivity, assessed population cell cycle kinetics of cultured KS, and documented their predominant albeit heterogeneous endothelial phenotype and enhanced proliferative potential.

MATERIALS AND METHODS

In 9 HIV-seropositive patients with AIDS, portions of punch or excisional biopsies of KS cutaneous lesions (including patch, plaque, and tumor varieties) were obtained under sterile conditions. The samples were coded KSUA 1 through 9. Representative portions of the tissues were fixed in 10% buffered formalin for light microscopy or half-strength Karnovsky's fixative for electron microscopy. The remaining portion was stored in M5 medium (1 part HAM'S F12 to 1 part DMEM) (Sigma) supplemented with 25mM HEPES 10% fetal bovine serum (FBS) (Hyclone) pH 7.2 at room temperature until processing, between <1 to 18 hours. Skin samples for cultures were trimmed of epidermis, minced to $1mm^3$, and digested in 200 U/ml collagenase type IV (Sigma) in magnesium and calcium free phosphate buffered saline (PBS-A) for 30 min at 37°C. Cell suspensions were pelleted and washed in FBS containing M5, resuspended in M5 10% or 20% FBS, seeded to Falcon T25 flasks, and routinely cultured at 37°C in a 7.5% CO_2 humid atmosphere waterjacketed incubator. Medium renewal or routine trypsin passage [1 to 5 minute incubation at 37°C in 0.2% trypsin (Sigma), 0.1% EDTA followed by neutralization with an equal volume of M5 20% FBS] was carried out weekly.

Endothelial cells derived from human omentum, umbilical vein, coronary vessel and recurrent lymphangioma of the upper extremity were isolated and propagated similarly and served as non-KS human endothelial cell controls.

Each culture flask was examined using an Olympus inverted light microscope with attached 35mm camera documenting changes in cell morphology, attachment or sprouting and branching (*in vitro* angiogenesis). Cells grown on coverslips were fixed in half-strength Karnovsky's solution or 3.7% formalin and processed for scanning electron microscopy (SEM) (ETEC autoscan) or fluorescence microscopy (FM) (Olympus epifluorescence).

Transmission electron microscopy (TEM) (Phillips CM 12S) was used to examine cells grown as monolayers for intercellular junctional complexes, density and morphology of surface microvilli, abundance, orientation and characteristics of intermediate and plasma membrane associated microfilaments, micropinocytotic vesicles, fenestrae or pores, Weibel-Palade bodies, cytoplasmic organelles, and basement membrane as well as specific evidence of inclusion bodies, viral particles, or syncytia formation.

Paraffin-imbedded tissue sections were studied using immunohistochemical stains for Factor VIII-related antigen (F8RAg) and with Ulex europaeus agglutinin (UEA). F8RAg was determined by the peroxidase-antiperoxidase (PAP) method. Briefly, deparaffinized sections were treated with 0.1% pronase for 10 min and washed with PBS. The sections were then sequentially treated with rabbit anti-F8RAg, swine anti-rabbit gamma globulin, and PAP reagent. The site of peroxidase localization was visualized by developing slides in 0.3% 3'-,3'-diaminobenzidine (DAB). UEA staining was performed by the 2-step method of exposure to biotin labeled Ulex lectin followed by avidin-horseradish peroxidase and development in 0.3% DAB. Immunoreactivity was assessed without knowledge of flow cytometry (FCM) results (*vida infra*) after assigning each biopsy specimen to a specific histologic stage as previously described (8). Stage 1 comprised dissecting lymphatic-like channels and/or variable proliferation of small blood vessels and oval endothelial cells. Stage 1t2 displayed partial microvascular loss, mild sclerosis, and the appearance of short spindle cell aggregates 3-4 cells thick. None of our patient material showed the spindle cell nodule characteristic of Stage 2.

Fluorescent labeling of cultured cells was performed on monolayers for FM or for FCM on trypsin released cell suspensions frozen in M5 with 20% FBS and 10% dimethylsulfoxide (DMSO), quick thawed, washed in PBS-A with 0.1% bovine serum albumin (BSA), and labeled at 4°C. F8RAg and angiotensin
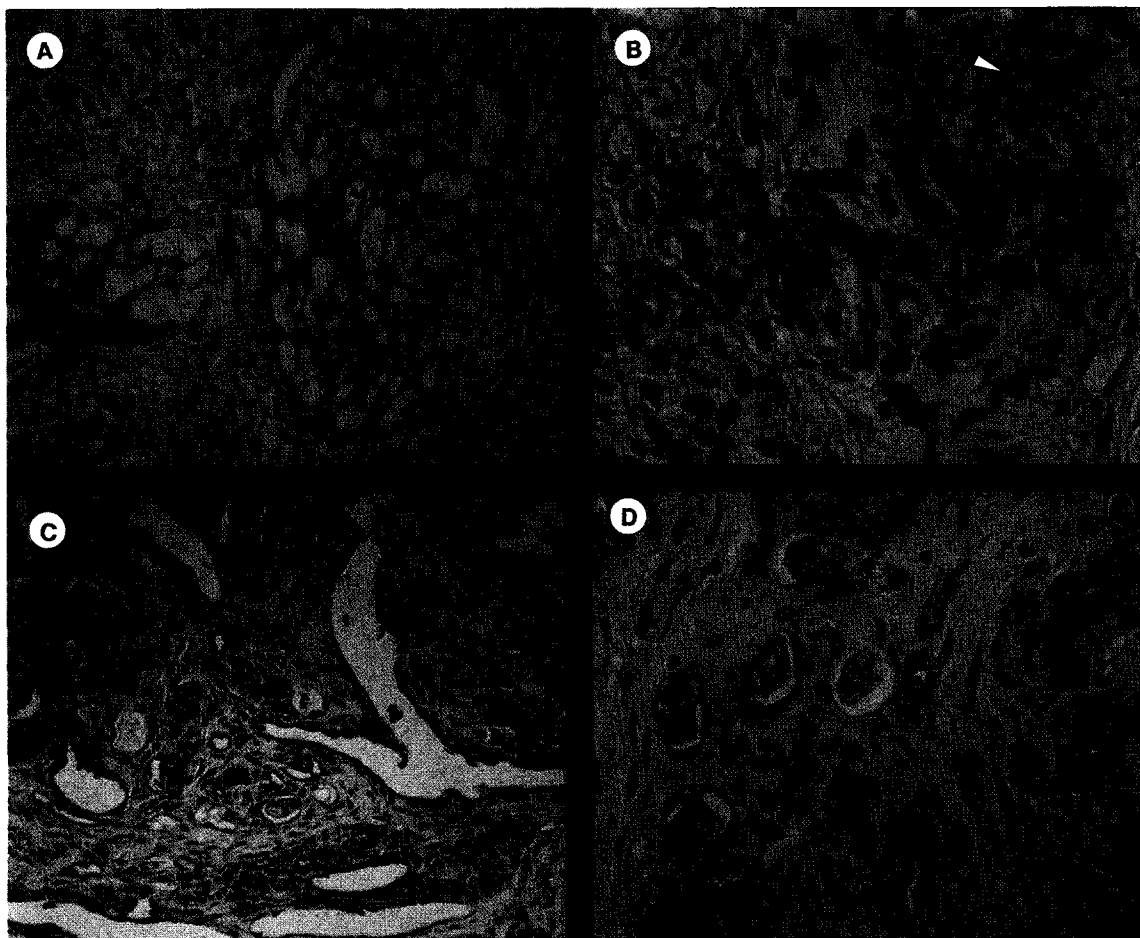
Fig. 1. Immunohistochemical staining of paraffin sections of original tissue from AIDS-Kaposi sarcoma skin biopsies. (A) Glomeruloid structure with variably stained endothelial lining cells to Factor VIII-related antigen (F8RAg) and (B) diffuse extracellular deposition of F8RAg (arrow). Note that collagen bundles traverse these aberrant clefts which are likely lymphatic in origin. (C) Dilated and slit-like vascular channels staining moderately positive for Ulex europaeus ligand (UEA) and (D) surrounded by fibrous tissue and spindle cells largely negative for UEA. Note red blood cells in the lumen of the microvessels, which are probably postcapillary venules. Whereas F8RAg staining was uniformly observed in the endothelial lining of larger vessels, the dilated channels with more attenuated endothelium stain variably. Despite spindle cell negativity for F8RAg and UEA (A-D), there is distinct pericellular staining with F8RAg. A,C Original magnification — x100; B,D — original magnification x250.

converting enzyme (ACE) antibody (Affinity, Deerfield, IL) staining was carried out using a three-step procedure where rabbit anti-F8RAg (Dako, Carpinteria, CA) or rabbit anti-ACE labeling was followed with biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA) and finally by fluorescein isothiocyanate (FITC) labeled avidin (Vector). UEA staining with biotinylated UEA (Vector) was followed by FITC labeled avidin (Vector) in a two-step immunofluorescent procedure. A final propidium iodide nuclear staining for cell versus debris identification was carried out overnight with 50μg/ml in PBS-A and 0.1% BSA at 4°C. Proliferating cell nuclear antigen (PCNA) (Oncogene science, Uniondale, NY) analysis was a one-step procedure where cellular isolates were exposed to FITC
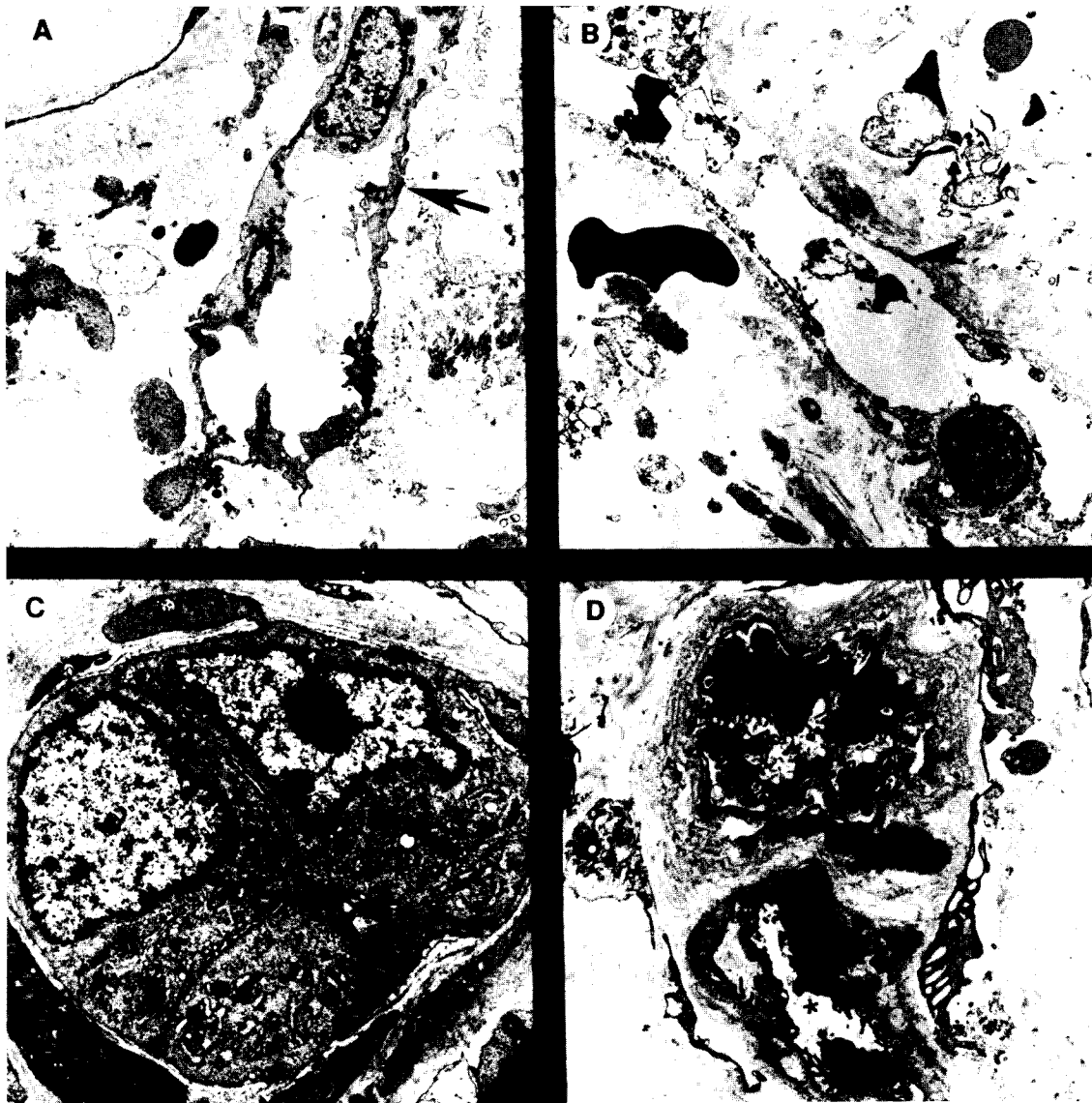
Fig. 2. Transmission electron micrographs of original tissue AIDS-KS biopsies. (A) Typical lymphatic endothelial-lined slit demonstrating overlapping junctions (arrow) and normal appearing nuclei with endothelium forming a continuous vascular cover. Junctional complexes appear slightly thickened. Collagen and elastin bundles are abundant. (B) Vascular slit conspicuously lacking an intact continuous endothelial cell lining. The endothelial lining cells exhibit perinuclear chromatin condensation. Although overlapping junctions are present, they lack definition. Portions of the slit are completely devoid of endothelial coverage (arrowhead). The lumen and adjacent interstitium contain an occasional red blood cell. Elastin and collagen bundles are prominent. (C) Vessels showing thickened (plump) endothelial lining cells, in this instance consisting of four plump cells. Note reduction in pericyte sheath, dissociation of the basement membrane trilayer, and dilated rough endoplasmic reticulum. (D) A more bizarre vessel showing little or no pericyte sheath, absence of well-formed junctional complexes, a thick disorganized basement membrane and numerous pinocytotic or endocytotic vesicles (asterisk) (inset). Original magnifications A—x2800, B—x3000, C—x3300, D—x3810.
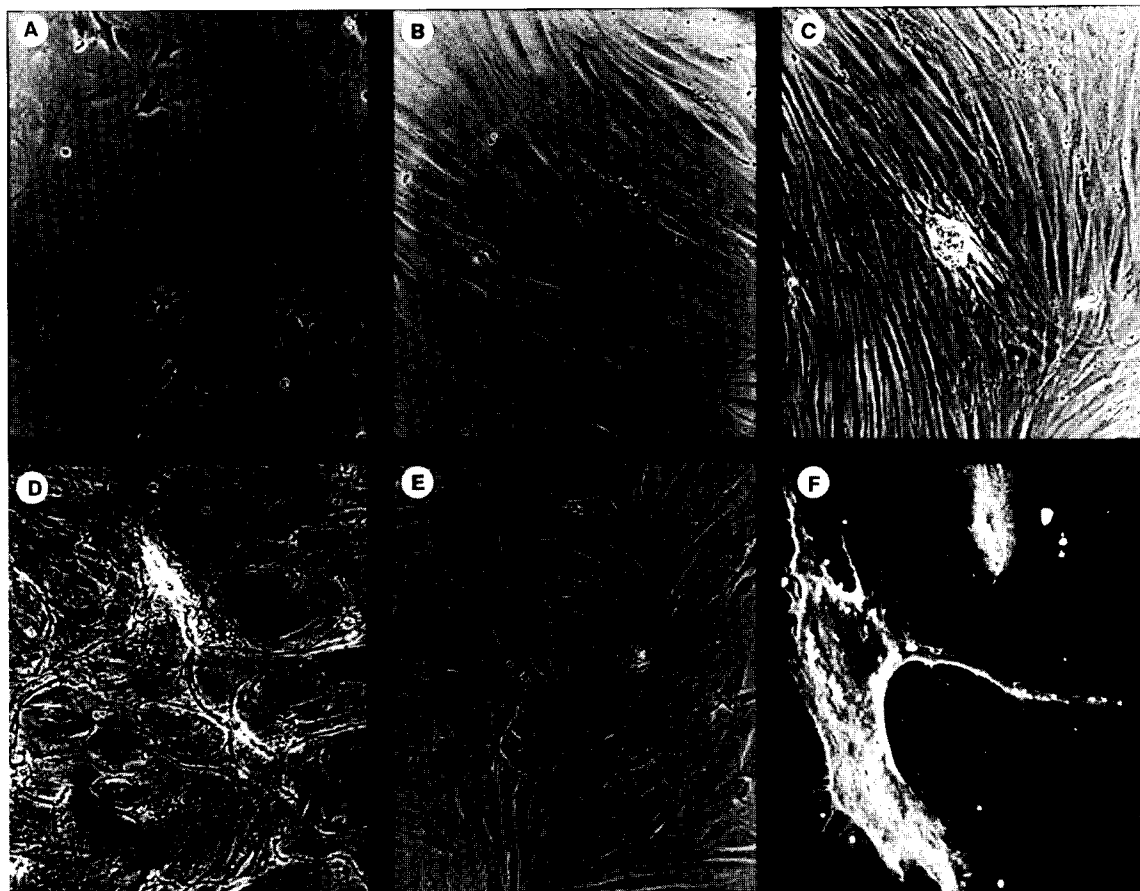
Fig. 3. Inverted light-phase contrast photomicrographs (A-F) of cultured KS cells. (A) stellate morphology and variable cell thickening, (B) spindle morphology showing a thickened profile, (C) spindle cells with nodular eruption, (D) fibrinous deposits with nodular protrusion, (E) mixed morphology with a stellate to polygonal thickened cell focus more centrally surrounded by bipolar, spindle-like cells. (F) Scanning electron micrograph of a single migratory cell showing few surface protrusions characteristic of endothelium in vivo with filopodia-like attenuations and a more stellate appearance. A-F, original magnification x33; H—bar=10 micron.

conjugated antibody, washed, and analysed by FCM. Immunophenotypic analysis consisted of subjective FM where approximately 200 cells were scored for positivity and FCM histogram dependent percent positivity evaluation of up to 10,000 FITC and propidium iodide dual labeled cells. FM high power fields were scored for marker positive events until approximately 200 cells were counted. FCM consisted of gated collection and storage of emission wavelengths of the 520±22nm (FITC labeled antibody) and 610±10nm (PI labeled DNA) ranges using a custom manu-

factured FCM Cytomutt (Howard Shapiro, M.D., P.C., Cambridge, MA) argon ion laser instrument (9).

Cultures for cell cycle analysis were held at maximum confluence (quiescence) (10) with routine medium changes for a minimum of 4 weeks prior to analysis. DNA compartmental analysis was determined using FCM. Briefly, after trypsin harvest and hypotonic permeabilization of the washed cells in 0.1% sodium citrate and 0.1% nonident P40 (Sigma) and double stranded RNA elimination with a 30 minute incubation at room temperature in
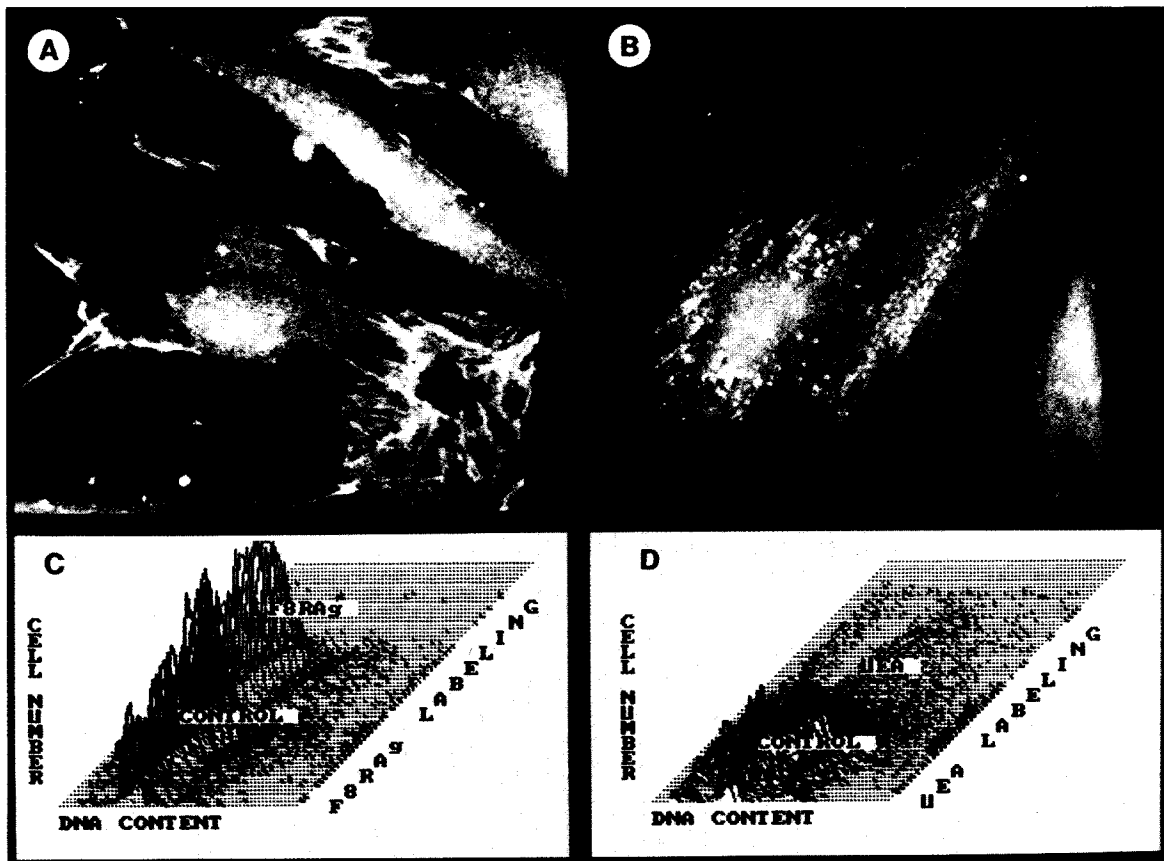
Fig. 4. Cultured Kaposi sarcoma cells. (A) fluorescence micrograph with intense decoration of extracellular matrix by F8RAg. (B) Fluorescence micrograph of UEA staining showing intense perinuclear region consistent with UEA binding. (C) Flow cytometric histogram corresponding to F8RAg marker decoration shown in (A). Peak left front (blue) is control propidium iodide and secondary antibody with fluorescein isothyiocynate (FITC) staining of cells but without primary antibody to F8RAg. Peak right rear (red) is anti-F8RAg stained cell population showing 88% positivity above that of control. (D) FCM corresponding to UEA marker decoration shown in (B). Peak left front (blue) is control and right rear (red) represents UEA positive cells or in effect a 70% positivity above control. See also Table 1 for summarized data on other culture specimens.

1mg/ml ribonuclease (Sigma), the nuclei were stained by the addition of 50µg/ml PI, and routine doublet corrected gated histogram analysis was done of the percent cells in the G_1, G_2M, and S cell cycle phases using Mod-Fit software (Verity House, Topsham, ME).

Cultured cells (10^6 cells in 0.5ml PBS-A) from two KS lesions and PBS-A alone were injected subcutaneously into the back of the neck of 6 athymic nu/nu nude mice, which were observed for 8 weeks and thereafter killed for autopsy.

RESULTS

Histologic staging of excised cutaneous KS lesions conformed in these 9 patients to either Stage 1 or 1t2. The endothelium lining smaller vascular channels and thin-walled clefts was typically positive for F8RAg and UEA *(Fig. 1)*. Whereas the spindle cell component was usually negative for these markers, there was often extracellular deposition of F8RAg adjacent to these cells *(Fig. 1B)*. Transmission electron microscopy

(TEM) of microvascular elements *(Fig. 2)* displayed numerous plump endothelial lining cells, a thickened disorganized basement membrane, loss of cell contacts and junctional complexes, and discontinuity of the pericyte envelope. Microvessels compatible with lymphatic capillaries sometimes contained red blood cells and showed variable degrees of endothelial cell degeneration characterized by cell loss and discontinuous lining *(Fig. 2B)*. No viral particles, inclusion bodies or syncytial formation were seen. A rare Weibel-Palade body was seen in the endothelial lining cells. Cytoplasmic organelles and intermediate filaments were unremarkable except for dilated endoplasmic reticulum. Anchoring filaments were not detected.

Initial growth was focal with interspersed migratory endothelial-like cells. At confluence, cells *(Fig. 3A-F)* were spindle *(Fig. 3B)* to stellate *(Fig. 3A,F)* and thickened. Prominent migratory appearing cells *(Fig. 3A)* and filamentous deposits developed after pro-tracted culture periods *(Fig. 3E)*, up to more than three years, with occasional foamy *(Fig. 3D)* and vacuolated cells. Endothelial cell thickening and at times early transitory nodule formation was occasionally observed at various sites *(Fig. 3C)*. Fluctuations in cell morphology within flasks appeared unrelated to the degree of confluence. A more polygonal shape was exhibited by some cells regardless of confluence level and more bipolar to spindle or stellate morphology in other cell popula-tions. Although spontaneous transdifferen-tiation of individual cells was not clearly demonstrated, as foci appeared, cells on the leading edge were seen to become more spindle-like and at confluence to return to a more polygonal or stellate morphology.

Cultures screened by inverted light and scanning electron microscopy and by immunohistochemical analysis for endothelial markers (F8RAg, UEA, and ACE) revealed populations consistent with the phenotypic profile of non-KS endothelium. In addition, prominent extracellular deposition of F8RAg noted in the original KS tissue *(Fig. 1B)*, was
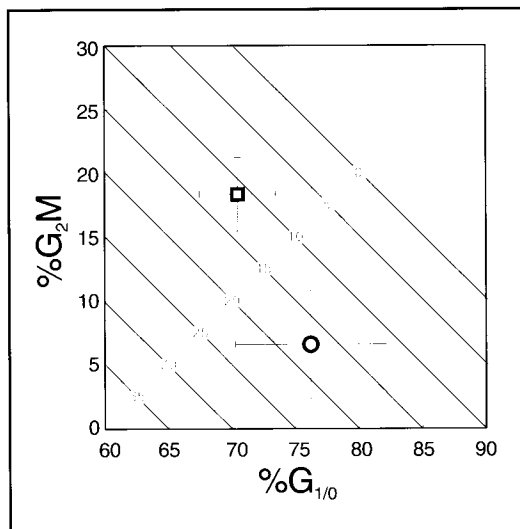

Fig. 5. DNA cell cycle analysis of quiescent cultured KS and non-KS cells. Diagonal lines represent S phase percentages. O=non-KS endothelial cells. □=KS cells. Data points represent mean±SE. Note the increase (p<0.04) in G_2M in quiescent KS cells.

also conspicuous in culture *(Fig. 4A)*. Although varying from culture to culture, FM and FCM analyses of the immunohisto-chemical profile of the KS cells *in vitro* corresponded closely to cell populations seen in the original KS tissue, consistently exhibiting prominent endothelial marker positivity for F8RAg, UEA, and ACE *(Table 1 and Fig. 4A-D)*.

DNA cell cycle compartment analysis *(Fig. 5)* showed quiescent KS cells with heightened G_2M phase percentage compared to cultured non-KS endothelium. Both KS and non-KS endothelial cells showed low doublet contamination with only a single omental microvascular culture requiring statistical correction. PCNA levels in AIDS-KS cells were increased at confluence induced quiescence.

After inoculation of cultured KS cells into nude mice, no tumors were detected during an 8 week period. Mice appeared active, alert, and otherwise healthy prior to sacrifice and negative autopsy examination.

DISCUSSION

AIDS-associated KS lesions yield a phenotypically heterogenous cell population which over successive generations in continuous culture culminate in a subpopulation consistent with an endothelial origin as reflected by endothelial antigen specific fluorescent antibody staining characteristics. These findings mirror those of the original tissue sections, which exhibited a phenotypic heterogeneity nonetheless characteristic of normal lymphatic and/or microvascular endothelium (6,7). While some suggest (11) that lymphatic endothelium exhibits a unique immunohistochemical staining pattern distinct from blood vascular endothelium on tissue section (e.g., negative for F8RAg), these distinctions are variable, quantitative rather than qualitative (12-14), and not persistent in culture (7,15). Although Weibel-Palade bodies, thought to represent storage sites for F8RAg, were not seen in the cultured KS cells in contrast to other cultured endothelial cells and are

exceedingly scant in KS tissue sections, recent findings indicate that this marker is unreliable for identifying endothelium both in KS vasculature and also in cultured endothelial cells undergoing transdifferentiation (8,16,17).

Whereas F8RAg deposition in the extracellular matrix surrounding spindle cells and vascular slits in KS tissue sections might be attributable to increased transcapillary leakage of plasma across highly permeable KS microvessels (17,18), it is noteworthy that similar extracellular F8RAg deposition is also detected in cultured KS cells, a finding suggestive of dysregulated von Willebrand factor storage and secretion by dysfunctional KS endothelium. Thus, rather than heightened F8RAg extravasation from enhanced plasma leak due to basement membrane breakdown, alteration of endothelial cell junctions or fenestrae, or even endothelial cell death, disturbed endothelial-extracellular matrix interactions involving F8RAg production, storage and extracellular release seem more likely (16). Indeed, a similar shift to

TABLE 1
Endothelial Cell (EC) Marker Percent Positivity of
Cultured KS and non-KS Cells Determined by Flow Cytometry

Cell Source	Marker (%+)		
	F8RAg	UEA	ACE
KS-EC			
1	66	43	87
2	77	71	83
3	61	51	–
4	72	42	–
5	55	–	50
6	58	28	55
7	74	25	41
8	74	25	63
9	64	–	38
Mean ± SE	64±3	40±9	81±9
Non-KS-EC			
Mean ± SE	72±11	55±17	73±7

F8RAg=Factor VIII-related antigen; UEA=Ulex europeaus agglutinin;
ACE=angiotensin-converting enzyme

F8RAg extracellular deposition has been described in isolated cultures of transdifferentiating endothelial cells (17), where such an altered metabolic and functional state is not attributable to a compromised vascular barrier.

Attempts to accurately quantitate cell type specific markers by fluorescent and light microscopy are more subjective than flow cytometric methods and often lead to sampling error. Thus subtle lightly positive staining or small, albeit focally distinct, positive-staining cell subpopulations are not consistently appreciated among observers. FCM, on the other hand, accurately and without observer bias characterizes and quantifies the staining properties of cells (9). Unlike FM, which measures hundreds of cell events over several minutes, FCM analyzes thousands of cellular events per second yielding highly accurate determi-nations of percent positivity *(Fig. 4, Table 1)* (19). Nonetheless, in the present study, as has been previously described (19), the statistical agreement between the calculated endothelial cell positivity for each cell line using FM and FCM was high, further validating FCM-based endothelial immunophenotypic identification procedure, which has the additional potential for cell sorting and ultimately a more detailed characterization.

The possibility remains of imprecise identification of KS cells isolated from differing subpopulations within a KS lesion (19-23). It is worth noting, however, that several groups (25-27) have documented a characteristic endothelial cell morphology and immunostaining of cultured KS cells from both AIDS and non-AIDS associated lesions. Moreover, definitive identification of exfoliated KS cells among desquamated pleural effusion cells rather than solid tissues raises serious questions as to the relevance of effusion-derived KS cells (28). Phenotypic differences among cell lines may relate to the tissue of origin (e.g., exfoliated pleural cells compared with skin lesions), culture conditions (e.g., virally conditioned media or added growth factors) or isolation techniques (e.g.

collagenase versus trypsin). In isolating a transdifferentiated or putatively transformed phenotype, however, it seems desirable, insofar as possible, to avoid adding undefined exogenous factors to culture media before establishing a basic *in vitro* phenotype in defining a cell population. We have succeeded in isolating morphologically, immunohistochemically, and DNA cell cycle kinetically similar cell types from all nine KS specimens with only minor variations in percent of typical endothelial cell marker positivity for each cultured population.

Although, to date, the spindle cell as defined in the *in vivo* tissue environment is often touted as the KS specific cell of origin, it is noteworthy that the spindle cell is not histologically prominent in early lesions (Stage 1) where glomeruloid structures predominate (1,29). Moreover, the spindle cell itself may merely represent a phenotypically altered transdifferentiated endothelial cell (1,8,17, 30,31), which has lost its endothelial marker positivity due in part to an altered basement membrane for attachment. In support of the concept of endothelial phenotypic heterogeneity, the endothelial cells lining the lumen of the lesional vascular slits exhibit weak and variable staining for endothelial markers whereas larger thick-walled vascular channels show nearly uniform positivity for endothelial markers. An immunophenotypic distinction between lymphatic and blood vessel endothelium is unclear with F8RAg in the former staining overall less intense and granular and tending to be more associated with surrounding extracellular matrix (12-14). Any distinction between these two microvascular cell types may actually disappear as they converge, particularly *in vitro*, and in some disorders (12-15).

Although the *in vivo* proliferative and neoplastic potential of KS remains unclear, the overall appearance, extracellular F8RAg deposition, and proliferative potential of the cultured KS cell is consistent with a transdifferentiated or transformed endothelial cell type (17,31), which is also reflected in elevated

G_2M phase and in elevated PCNA levels at contact inhibited quiescence. We interpret the G_2M elevations as a readiness for rapid entry into the cell cycle (32).

Despite longevity in culture, AIDS-KS cells herein described (as well as AIDS-KS isolates previously reported by others) fail to produce tumors in nude mice. On the other hand, transitory angiogenic lesions, also found after control non-KS cell injections, may be demonstrable at 7 days (personal observation). These findings, in conjunction with inability of cultured KS cells to stimulate production of KS specific monoclonal anti-bodies by conventional hybridoma technology (33), are consistent with a proliferative but not necessarily neoplastic process. Ultrastructural changes observed by TEM in KS tissue sections (namely plump endothelium, disorganized basement membrane, loss of cell contacts and junctional complexes, and discontinuous pericyte envelope) are also features of wound healing and repair (34), a phenomenon also characterized by endothelial cell transdifferentiation, migration and proliferation. On the other hand, an abnormal proliferative component indicative of cell cycle dysregulation is not readily detectable in KS lesions *in situ* (35). However, cell cycle analysis of mixed populations is extremely difficult since a small percentage of abnormal cells may be overwhelmed by normal cells obscuring subtle cell subpopulation abnormalities.

In conclusion, AIDS-KS cells in long-term culture retain features consistent with an endothelial cell phenotype and which are shared both by fixed and sectioned KS lesions and cultured non-KS lymphatic and blood vascular endothelia. These AIDS-KS cells also display a proliferative potential consistent with transdifferentiation/transformation maintaining slow continuous growth *in vitro* for more than three years. KS cell lines, such as described in this report, may be useful to screen putative promoters or suppressors of KS lesional growth including an array of growth factors, cytokines, virally infected cells and cell products, and extracellular matrix factors and also in the identification of other yet to be discovered AIDS cofactors.

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