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Rapid Communication

AIDS-associated Kaposi's Sarcoma-derived Cells in Long-term Culture Express and Synthesize Smooth Muscle Alpha-actin

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Spindle-shaped cells from Kaposi's sarcoma lesions (AIDS-KS cells) were cultured for long periods in the presence of conditioned medium from activated CD4-positive T cells (HTLV-II infected transformed nonvirus producer) and characterized under* in vitro *conditions. To investigate a possible vascular origin, AIDS-KS cells were analyzed for the presence of smooth muscle α-actin, a differentiation marker for vascular smooth muscle cells. Immunofluorescence studies using a monoclonal antibody for smooth muscle α-actin demonstrated positive staining of the AIDS-KS cells (KS-3 and KS-4) but not by endothelial cells or fibroblasts. Northern blot analysis using an oligonucleotide probe unique for human smooth muscle α-actin indicated the expression of this gene by AIDS-KS cells. Similar analysis of biopsies from the KS lesion showed that in addition to the staining of smooth muscle cells associated with the blood vessels, the tumor-related spindle cells also stained positively. These cells were also analyzed for the expression of different growth factor genes. The platelet-derived growth factor (PDGF) A-chain gene was expressed at a moderate level. The insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-2) genes were not overexpressed in relation to control cells. These data suggest that the analyzed AIDS-KS cells may be smooth muscle-like cells and therefore of vascular origin. Based on these results as well as previous reports, we speculate that cells of the immune system may regulate growth of cells in the vascular wall by a novel pathway. (Am J Pathol 1991, 139:1251–1258)

Kaposi's sarcoma (KS) lesions have a complex histology characterized by abnormal vascularization and the presence of spindle cells, fibroblasts, and infiltrating white blood cells. Capillary endothelial cell proliferation and spindle-cell proliferation appear early in KS lesions and can be found in all subsequent stages of development.[1] The origin of the spindle cell is unclear.

Earlier investigations of the origin of Kaposi's sarcoma-derived cells (KS cells) were hampered by the complex nature of the lesions and by the lack of appropriate culture methods for these cells. Recently, a procedure was developed for establishing AIDS-KS cells in long-term culture.[2] These cells were initially derived from lung biopsies and pleural effusions from AIDS patients with Kaposi's sarcomas.[2,3] They were cultured in the presence of growth factors released by the activated T-cells infected with human T-lymphotropic viruses type I or II (HTLV-I or HTLV-II) or with human immunodeficiency virus type 1 or 2 (HIV-1 or HIV-2).[2]

Previous studies indicated that AIDS-KS cells in culture produce mitogens (e.g., interleukin-1 and bFGF) that support their own growth (autocrine activities) as well as the growth of human endothelial cells and fibroblasts

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(paracrine activities).[4] These results suggest that spindle cells may stimulate the abnormal vascularization in KS lesions.

AIDS-KS cells display some features that are characteristic of endothelial cells and some that are characteristic of other tissues. For example, AIDS-KS cells in long-term culture lack the Weibel-Palade bodies and are negative for factor VIII,[3] characteristic of vascular endothelial cells. This marker is also absent in spindle cells or in primary KS tissue. On the other hand, AIDS-KS cells are positive for a lectin binding site specific for *Ulex europaeus* and enhanced uptake of acetylated low-density lipoprotein (Ac-LDL), marker which distinguish endothelial cells from other cell types.[5]

To further investigate a possible vascular origin of the spindle cells from AIDS-KS lesions, we looked for smooth muscle α-actin, which is a differentiation marker characteristic of vascular smooth muscle cells and pericytes. Immunofluorescence studies demonstrated that AIDS-KS cells were positive for vascular smooth muscle α-actin. Additionally, Northern blot analysis using an oligonucleotide unique for human smooth muscle α-actin confirmed the expression of this gene in AIDS-KS cells, but not in endothelial cells or fibroblasts. The presence of smooth muscle α-actin in primary KS tissue was also demonstrated using indirect immunoperoxidase technique.

This is the first study that indicates that AIDS-KS cells can in long-term culture have specific markers for human smooth muscle cells. These cells may be originally derived from a pluripotential mesenchymal (vascular) cell type that can differentiate into a smooth muscle cell-like or pericyte-like form, such as the spindle cell in the more specialized connective tissue from Kaposi's sarcoma lesion.

Materials and Methods

Cells

KS-3 and KS-4 cell lines were initially isolated from lung biopsies and cultivated as long-term cultures.[3] Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 15% calf serum (HyClone, Logan, UT), essential and nonessential amino acids (Gibco) and 20% conditioned media from HTLV-II infected CD4 + T lymphocytes. Cells were maintained in an humidified 37°C and 10% CO_2 atmosphere, and medium was replaced twice weekly.

Antibodies and DNA Probes

Mouse monoclonal antibody 1A4 directed against a synthetic decapeptide specific for smooth muscle α-actin was obtained from Sigma (St. Louis, MO).[6] Fluorescein-conjugated and rhodamine-conjugated goat anti-mouse IgG were obtained from Cappel Laboratories (Cochranville, PA).

The following DNA probes were used: 1) smooth muscle α-actin; a 25-mer oligonucleotide (AGTGCT-GTCCTCTTCTTCACACATA, codons 1 to 8) unique to the human smooth muscle α-actin gene,[7] 2) PDGF-A; a 0.7-kilobase (kb) RsaI human cDNA fragment released from plasmid pPGF-1,[8,9] 3) PDGF-B; a 2.0 kb Bam HI human cDNA fragment released from plasmid pMVW-2,[10] 4) IGF-1; a 0.2 kb Sau3A human cDNA fragment released from plasmid pI-Sau3A203E[11] and provided by J. Hoppener (State University of Utrecht, Netherlands), 5) IGF-2; a 0.3 kb Pvu II/Bal I human cDNA fragment released from the plasmid pIGF-II/1[12] and provided by K. Rosen (Children's Hospital, Boston, MA), 6) hst; a 0.5 kb Sac I/Eco RI human cDNA fragment released from the plasmid KS3[13] and provided by P. Powell (Children's Hospital, Boston, MA).

Indirect Immunofluorescence

For the immunofluorescence studies, AIDS-KS cells and control cells were plated on gelatinized Lab-Tec microslides (Nunc Inc., Naperville, IL) at 1–2 × 10^4 cells/well and were grown to near confluency (3 days). The medium was removed and the slides were rinsed two times with phosphate-buffered saline (PBS) (0.15 M NaCl, 0.05 M sodium phosphate, pH 7.4), fixed with cold methanol for 5 minutes at −20°C, and rinsed again in PBS. The slides were subsequently washed with PBS containing 0.2% Triton X-100 and 0.1% bovine serum albumin (PTB) and incubated with anti-smooth muscle α-actin antiserum (diluted 1:400 in PTB) over 60 minutes at 4°C. After washing two times with PBS, slides were incubated with the affinity-purified fluorescein- or rhodamine-conjugated goat anti-mouse IgG (diluted 1:200 or 1:75 in PTB, respectively) over 60 minutes at 4°C. After washing several times with PBS, slides were covered with glycerol and mounted for immunofluorescence microscopy. Micrographs were taken with a photomicroscope (No. II, Carl Zeiss, Inc., Thornwood, NY) equipped for epi-fluorescence using a 16 and 40 X Zeiss Plan-Neofluor objective. Smooth muscle α-actin was visualized using the standard fluorescein or rhodamine excitation/emission filter combination and a color slide film (Scotch Chrome 640 T; 3M Inc., St. Paul, MN) for documentation.

Immunohistology

Biopsies of human skin tissues were fixed in formalin. Four micrometer thick sections were prepared with a mi-

crotome (Leitz-1512) on slides precoated with 3% Elmer's glue. The slides were heat-treated in 60°C overnight. The sections were deparaffinized with xylene and ethanol. The sections were also treated with 0.05% trypsin solution and rinsed with tap water and PBS. Sections were air dried, washed with PBS, and incubated in 0.1% sodium azide with 0.1% H_2O_2 in PBS for 15 minutes to inactivate endogenous peroxidase. After the washes in PBS, blocking was carried out with 10% normal calf serum (CS) in PBS for 30 minutes followed by incubation with the antibody to smooth muscle α-actin (3 μg/ml, diluted in 10% CS). After 60 minutes, sections were washed three times in PBS and incubated for 60 minutes with goat-anti mouse IgG peroxidase conjugated (Vector Laboratories, Burlingame, CA). After three additional washes, bound peroxidase was developed with 3,3′-diaminobenzidine (DAB; Sigma) for 5 minutes to produce a brown reaction product. Sections were counterstained with Mayer's hemalaun for 1 minute and embedded in aqua mount (BDH-Chemicals, Poole GB).

Preparation of RNA and mRNA Blot Hybridization

Total RNA from cultured cells was prepared as described[14] except that the protease digestion step was omitted. Electrophoresis, transfer, and hybridization with cDNA probes were performed as described.[15] For hybridization with the 25-mer oligonucleotide specific for smooth muscle α-actin, blots were prehybridized at 42°C in 4 × SSC (1 × SSC = 0.15M NaCl/0.015 M sodium citrate), 0,7% $NaDodSO_4$, 30% formamide, 5 × Denhard's solution, 5% dextran sulfate, 0.1% pyrophosphate, and 100 μg/ml denatured salmon sperm DNA over 2 hours. Hybridization was carried out in the presence of ^{32}P-labeled probe (1.2×10^6 cpm/ml) at 42°C for 16 hours. Blots were subsequently washed at 65°C with 2 × SSC, 1% $NaDodSO_4$, 0.1% pyrophosphate. Filters were air dried and exposed to Kodak XAR films with intensifying screens. Ribosomal RNA bands were indicated as size markers.

Results

Distribution of Smooth Muscle Alpha-actin in AIDS-KS Cells and Control Cells

To analyze a possible vascular origin of our isolated Kaposi's sarcoma cells, we used a monoclonal antibody against smooth muscle α-actin specific for the smooth muscle-alpha isoform of actin within a broad range of concentrations and in the presence of an excess of other actin isoforms.[6]

AIDS-Kaposi's sarcoma cells (AIDS-KS cells) from long-term culture, human vascular smooth muscle cells, human foreskin fibroblasts, and bovine aortic endothelial cells were plated on gelatinized microslides and subsequently stained with a monoclonal antibody specific for smooth muscle α-actin (Figure 1). In contrast to human foreskin fibroblasts and bovine aortic endothelial cells, AIDS-KS cells (KS-3 and KS-4) were morphologically similar to human vascular smooth muscle cells. Spread AIDS-KS cells showed a high degree of immunopositive stress fibers after staining with antibodies to smooth muscle α-actin that was also observed in human vascular smooth muscle cells (Figure 1a–d). The α-actin positive cells differed in the amount of smooth muscle α-actin from cell to cell (Figure 1a, 1b). In contrast to human cells, bovine vascular smooth muscle cells showed uniform staining of smooth muscle α-actin (not shown). On the other hand, human foreskin fibroblasts and bovine aortic endothelial cells (Figure 1e, 1f) showed a low, nonspecific diffuse staining pattern. No smooth muscle α-actin-containing stress fibers were detectable in these cell types.

Expression of Smooth Muscle Alpha-actin mRNA in AIDS-KS Cells and in Control Cells

To confirm our results from the immunofluorescence studies, we used an oligonucleotide as a probe for smooth muscle α-actin. The 25-mer oligonucleotide is unique for the smooth muscle α-actin gene and codes for the first eight amino acids of the protein.[7] To quantify the results described earlier, AIDS-KS cells and various other vascular cell types were examined for the expression of smooth muscle α-actin mRNA. Equal amounts of total cellular RNA were examined by Northern blot analysis. The expression of smooth muscle α-actin mRNA was detectable as a 1.8 kb transcript in human vascular smooth muscle cells and in AIDS-KS cells. Although our immunofluorescence studies produced similar patterns in KS-3 and KS-4 cells stained with anti-smooth muscle α-actin, Northern blot analysis demonstrated a difference in the expression rate between the two cell isolates. The reason for the lower rate of expression of the smooth muscle α-actin gene in KS-4 cells is unknown.

A low expression rate of this gene was detected in human foreskin fibroblasts, suggesting that our cells were contaminated with a low percentage of smooth muscle cells. Immunofluorescence studies confirmed that ap-

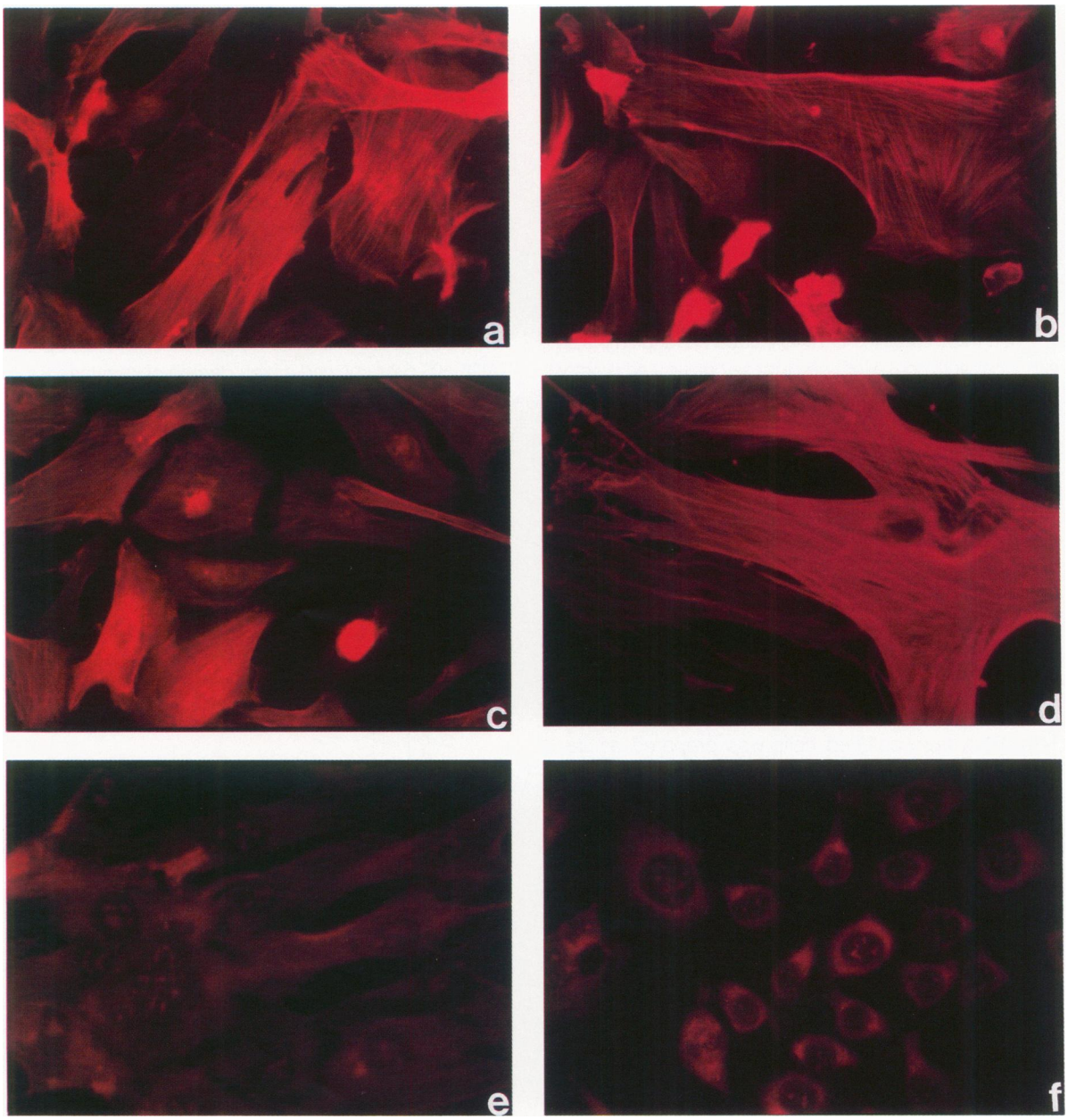

Figure 1. *Immunofluorescent staining for smooth muscle α-actin in KS-3* **(a, b)** *and KS-4 cells* **(c)**, *human vascular smooth muscle cells* **(d)**, *human foreskin fibroblasts* **(e)** *and bovine aortic endothelial cells* **(f)**. *AIDS-KS cells and human vascular smooth muscle cells brightly stained with anti-smooth muscle α-actin* **(a–d)**. *Stress fibers are clearly seen in the anti-smooth muscle α-actin positive cells and note that the degree of staining often differs from cell to cell* **(a)**. *Cultivated human foreskin fibroblasts and bovine aortic endothelial cells are negative for this antibody* **(e, f)**; *(×400).*

proximately 2 to 5% of our fibroblast cultures were smooth muscle cells (not shown). No expression of this gene could be detected in endothelial cells (Figure 2). Analyzed together, results from the immunofluorescence studies and Northern blot analysis both suggest that AIDS-KS cells in long-term culture express and synthesize a protein, which is a cell type-specific marker for smooth muscle cells.[6,16]

Distribution of Smooth Muscle Alpha-actin in Normal Skin and Primary KS Tissue

Given the fact that cultured AIDS-KS cells produce smooth muscle α-actin, we analyzed the distribution of these actin isoform in normal skin and in early KS lesions of the skin. In normal skin, only smooth muscle cells surrounding the blood vessels are strong positive by immu-

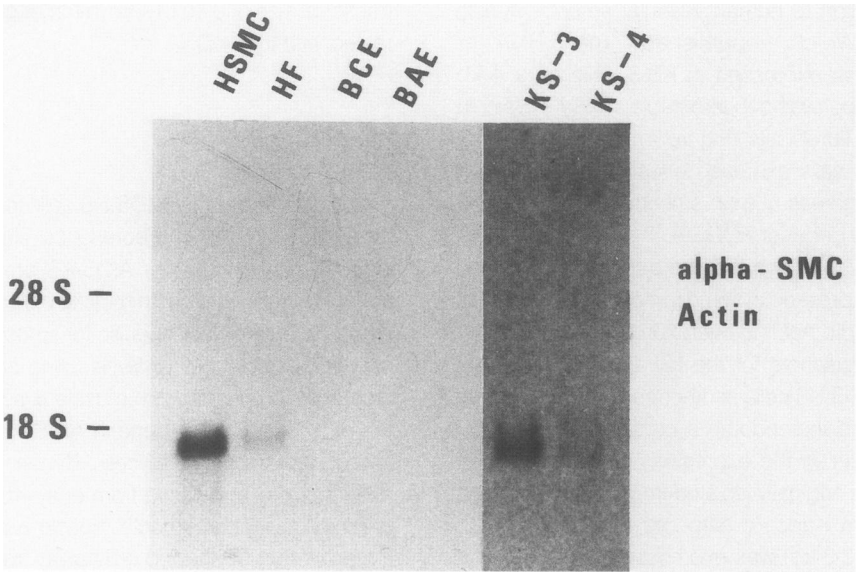

Figure 2. *Analysis of smooth muscle α-actin mRNA levels in AIDS-KS cells and other vascular cell types. In each lane, 5 μg of total RNA was fractionated in a 1.2% agarose gel containing formaldehyde, and transferred to a nitrocellulose membrane. 1.8 kb smooth muscle α-actin transcripts were detected by hybridization to a ^{32}P-labeled 25-mer oligonucleotide, a labeled DNA probe unique for the first eight codons of the smooth muscle α-actin gene (7). 1: human vascular smooth muscle cells. 2: human foreskin fibroblasts. 3: bovine capillary endothelial cells. 4: bovine aortic endothelial cells. 5: KS-3 cells. 6: KS-4 cells. Lanes are numbered left to right.*

noperoxidase staining (Figure 3a). However, spindle-shaped cells away from blood vessels stained also positive in biopsies from early KS lesions of the skin (Figure 3b). But, not all spindle-shaped cells are positive for this differentiation marker.

Expression of the PDGF Genes and Other Growth Factor Genes by AIDS-KS Cells

In previous studies we characterized the expression of different growth factor genes in AIDS-KS cells and found mRNA for aFGF, bFGF, TGFβ, interleukin-1a, and interleukin-1b.[4] AIDS-KS cells were further analyzed for other growth factors such as PDGF and IGF-1. Equal amounts of total RNA were analyzed by Northern blot analysis for the expression of PDGF-A, PDGF-B, IGF-1, IGF-2 and hst genes. As shown in Figure 4, AIDS-KS cells in long-term culture express the mRNA for the PDGF-A chain gene at a moderate level. Three PDGF-A transcripts of approximately 1.7, 2.3, and 2.8 kb were previously found in several human cells[8,17] and the sizes of three bands were almost identical to the sizes found in AIDS-KS cells and in human foreskin fibroblasts (Figure 4). The expression rate

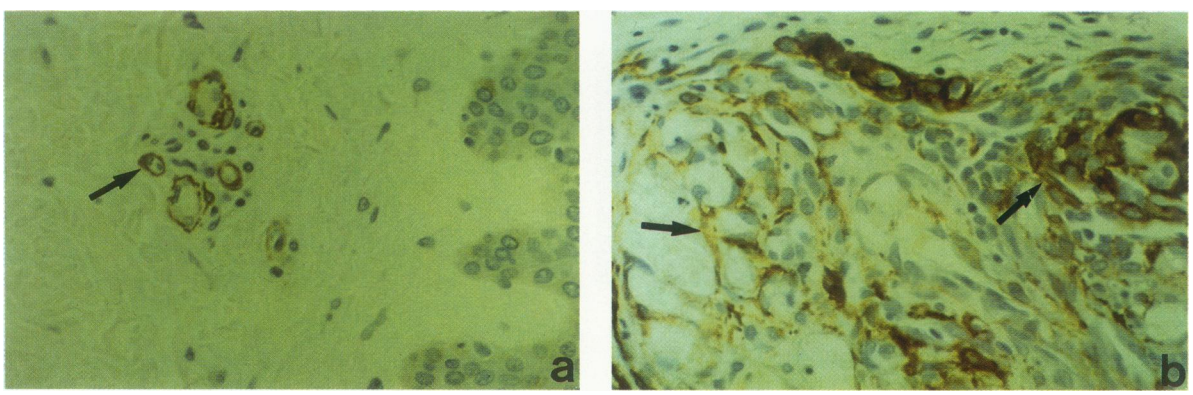

Figure 3. *Immunoperoxidase staining of smooth muscle α-actin in normal skin and skin with early KS lesions. Staining was performed on sections with a monoclonal antibody to smooth muscle α-actin. Sections were counterstained with Mayer's hemalaun.* **a**: *Normal skin: note that smooth muscle α-actin localizes only within the smooth muscle cells surrounding the blood vessels (×600).* **b**: *Early KS lesions of the skin: positively brown stained smooth muscle cells around the vessels and some of the spindle cells away from blood vessels are positive for smooth muscle α-actin (×600).*

is similar in relation to human vascular smooth muscle cells (Herbert Weich, unpublished). The mRNA for PDGF-B was also expressed in KS-3 cells as a 4 kb mRNA species, but was not detectable in KS-4 cells or in human foreskin fibroblasts (Figure 4). Interestingly, human endothelial cells express genes for both PDGF-A and PDGF-B, whereas human smooth muscle cells express mainly the gene for PDGF-A.[16,17,18,23,24]

Recently, it was shown that the genes for IGF-1 and IGF-2 are overexpressed in some tumors of smooth muscle origin.[19] We did not find overexpression of the IGF-1 gene. mRNA transcripts for the IGF-2 genes were not detectable in AIDS-KS cells or other control cells such as human fibroblasts and endothelial cells (data not shown).

We also analyzed the expression of the hst (kFGF) gene, which was originally isolated from human stomach cancer and from Kaposi's sarcoma, respectively.[13,20] The hst mRNA (3.6 kb) was expressed at a low level in KS-3 cells, whereas KS-4 cells and human foreskin fibroblasts were negative (data not shown). The transcript size is in agreement with earlier reports, showing a low expression of this gene in primary tumor cells and a major transcript size of 3 kb to 4 kb in tumor cells or cells transfected with tumor DNA.[13,21,22]

Discussion

These data show, that AIDS-KS cells from long-term culture have several characteristics similar to vascular smooth muscle cells: 1) AIDS-KS are morphologically similar to vascular smooth muscle cells; 2) AIDS-KS cells stain with an antibody specific for smooth muscle α-actin and; 3) Northern blot analysis using an oligonucleotide unique for human smooth muscle α-actin demonstrates the expression of this gene in AIDS-KS cells, but not in fibroblasts or endothelial cells. By using immunoperoxidase staining and tissue from early KS lesions we also demonstrated, that smooth muscle α-actin can be detected in spindle-shaped cells away from blood vessels.

Taken together, these results provide further support for a possible vascular origin of AIDS-KS cells.[3] Our results also demonstrate that these cells are not identical to normal vascular smooth muscle cells and may in fact

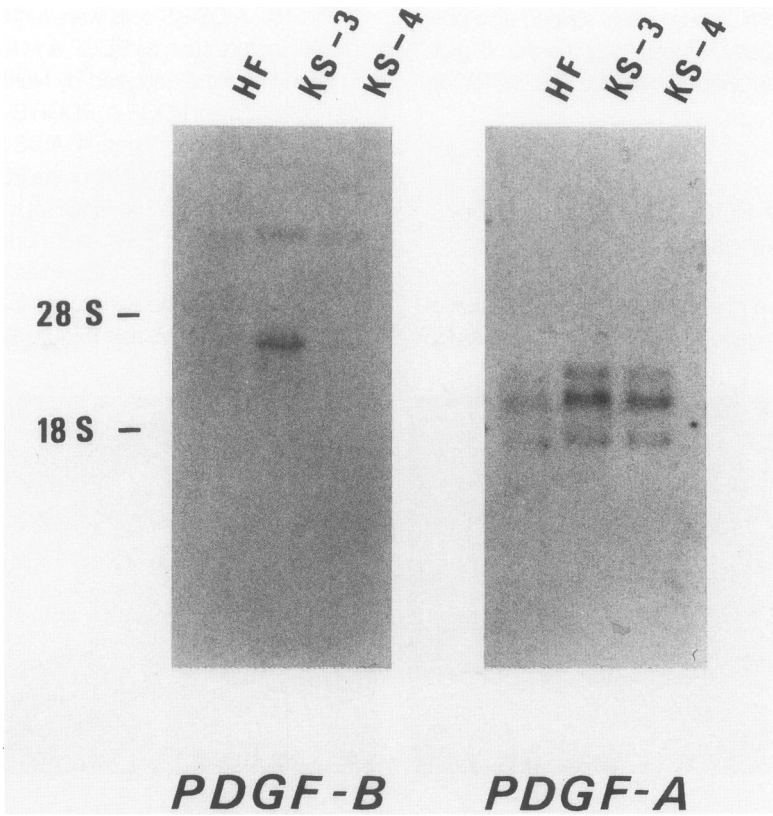

Figure 4. *Northern blot analysis of PDGF-B and PDGF-A mRNA expression in AIDS-KS cells (KS-3 and KS-4) and human foreskin fibroblasts. In each lane, 10 μg of total RNA was fractionated on a 1.2% agarose gel containing formaldehyde, and transferred to nitrocellulose membranes. The PDGF-B chain transcripts (PDGF-B) were detected by hybridization to a ^{32}P-labeled 2 kb Bam HI human cDNA fragment and the PDGF-A chain transcripts (PDGF-A) were detected by hybridization to a ^{32}P-labeled 0.7 kb Rsa I human cDNA fragment.*

represent a precursor version of undifferentiated smooth muscle. For example, our gene expression studies showed that some isolates of AIDS-KS cells are capable of expressing the gene for PDGF-B. This gene has a low expression rate in smooth muscle cells of the artery wall and in cultured smooth muscle cells, but is expressed at higher rates in embryonic smooth muscle cells from rat tissue.[18,23]

Even if smooth muscle α-actin is considered to be a differentiation marker for smooth muscle cells and pericytes,[6] it can be found in several other nonmuscle cell types, especially during various physiologic and pathologic conditions. For example, TGF-beta 1 can induce the expression of smooth muscle α-actin in rat capillary endothelial cells under certain conditions[25] and myofibroblasts synthesize smooth muscle α-actin under pathologic conditions and during wound healing.[26,27] Isolated stroma cells of the bone marrow are also positive for smooth muscle α-actin when taken into culture.[29] Studies of experimental models of nephritis confirmed that smooth muscle α-actin expression is a marker for mesangial cell injury and associated with cell proliferation.[30] In respect to these studies, we cannot exclude the possibility that another cell type than the vascular smooth muscle cell is the precursor cell type for our AIDS-KS cells. In addition, the selected culture conditions may have an effect on the induction of smooth muscle α-actin. The immunoperoxidase staining with early KS tissue also indicated that not all spindle-shaped cells of the lesions are positive for this actin isoform, which may also indicate that the positive staining of all our long-term cultured AIDS-KS cells can reflect the induction of the smooth muscle α-actin gene expression under the selected culture conditions.

Another difference between AIDS-KS cells and normal smooth muscle cells is that AIDS-KS cells contain a lectin binding site specific for *Ulex europaeus* as well as for acetylated low-density lipoprotein. Both markers are routinely used to distinguish endothelial cells from other cell types.[3,4] AIDS-KS cells also express and synthesize low level of interleukin-1a and interleukin-1b constitutively at high levels together with an altered form of bFGF which can support their own growth.[4] These characteristics are not reported for normal vascular smooth muscle cells.

Interestingly, AIDS-KS cells are stimulated by products of activated T-cells.[2] Therefore, we speculate that activated T-cells secrete a potent mitogen for AIDS-KS cells. Because AIDS-KS cells appear to have a smooth muscle-like origin (these results), activated T-cells mediate smooth muscle cell growth in response to balloon catheter-induced arterial injury.[31] These authors also demonstrated that smooth muscle proliferation *in vivo* was suppressed by inhibition of T-cell activation by cyclosporin A. These *in vivo* results, together with our previous reports[2,3,4] and the *in vitro* data described here, suggest a novel regulation of growth of cells in the vascular wall by cells of the immune system.

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