Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 7046–7050, August 1992
Medical Sciences

Expression of platelet-derived growth factor and its receptor in AIDS-related Kaposi sarcoma *in vivo* suggests paracrine and autocrine mechanisms of tumor maintenance

(*in situ* hybridization/immunohistochemistry/mixed-cell tumor/human immunodeficiency virus type 1)

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Communicated by Robert C. Gallo, April 28, 1992 (received for review August 26, 1991)

ABSTRACT **As previously described, proliferation of Kaposi sarcoma (KS)-derived cells *in vitro* is dependent on the presence of platelet-derived growth factor (PDGF). To test the hypothesis that PDGF may also be a major growth factor for KS cells *in vivo*, we performed *in situ* hybridization and immunohistochemical staining for PDGF and PDGF receptors in tissue sections of AIDS-related KS. The data suggest that KS consists of two types of tumor cells. (*i*) The main population are spindle-shaped cells with elongated nuclei (KS-s cells). They reveal a strong expression of PDGF β receptors but do not express the PDGF-A and PDGF-B isoforms. (*ii*) A minor population of KS cells express PDGF β receptor as well as PDGF-A and PDGF-B (KS-p cells). These cells are often grouped in whorls and surrounding vascular slits. They reveal spherical nuclei with evenly distributed chromatin and inconspicuous nucleoli. PDGF α receptor is not expressed in either form of KS cells. The results suggest that the isoforms of PDGF and the PDGF β receptor are differentially expressed in two different cell types in KS and that PDGF isoforms may contribute to the pathogenesis of KS.**

Platelet-derived growth factor (PDGF) is a major mitogen in human serum and consists of three isoforms (PDGF-AA, -BB, -AB). It is produced by endothelial cells, activated macrophages, aortic smooth muscle cells, mitogen-stimulated fibroblasts, and various tumor cell lines (1, 2). Binding characteristics of the various PDGF isoforms have revealed two distinct PDGF-binding proteins (α and β receptors) functioning as subunits in the active dimeric forms (3, 4). PDGF receptors are expressed in fibroblasts, smooth muscle cells, diverse tumor cell lines, and capillary endothelial cells (5–8) and in hyperplastic endothelial cells of glioblastomas (9). PDGF is involved in wound healing (10), abnormal proliferative processes such as arteriosclerosis (11) and rheumatoid arthritis (12), and cellular transformation (1).

Kaposi sarcoma (KS) is a rare mesenchymal tumor of low malignancy in elderly men. When associated with human immunodeficiency virus type 1 (HIV-1) infection, a more aggressive type of KS is observed. It is not clear whether KS is a true neoplasia or a hyperplasia. KS lesions may simulate a number of histopathological conditions in the skin—i.e., granulation tissue, hemangiomas, hemangiosarcomas, or fibrosarcomas (13, 14). The most characteristic feature of KS are spindle-shaped cells with elongated nuclei. They are abundant in the tumor, reveal a low mitosis rate, and are euploid (13, 14). Many authors believe that these so-called spindle cells are derived from endothelial cells of capillary or lymphatic origin, although staining for endothelial cell markers is inconsistent (13, 15–17). An origin from pericytes, smooth muscle cells, fibroblasts, and dermal dendrocytes has also been postulated (14, 18–20). In tissue culture, KS-derived cells resemble normal fibroblasts or smooth muscle cells and very often they are intermixed with cells expressing endothelial cell markers (16, 18, 21). Cultivated KS cells are negative for HIV-1 and many other viruses tested (22, 23). Nevertheless, HIV-1 Tat protein can stimulate the proliferation of KS cells *in vitro* (24).

KS expresses basic fibroblast growth factor (bFGF), fibroblast growth factor 5 (FGF-5), and interleukin 6 *in situ* (25, 26) and PDGF-A, PDGF-B, bFGF, acidic FGF, FGF-5, interleukin 1, interleukin 6, and granulocyte/macrophage colony-stimulating factor *in vitro* (26–29). Expression of the interleukin 6 receptor (*in situ*) and PDGF α and β receptors (*in vitro*) has been studied (26, 28).

Our previous studies on *in vitro* cultivated KS cells revealed that they were highly dependent on PDGF as a major mitogen (30). Therefore, we investigated the expression of PDGF and PDGF receptors *in situ* to elucidate further the role of this growth factor in the pathogenesis of the tumor. We show KS to be a mixed-cell tumor composed of two types of tumor cells: spindle cells expressing solely PDGF β receptor and cells expressing PDGF-A, PDGF-B, and PDGF β receptor. Mechanisms of autocrine and paracrine tumor growth promotion will be discussed.

MATERIALS AND METHODS

Patients. The group of HIV-infected donors included three patients in Centers for Disease Control (CDC) group IV (31) (patient 1, CDC IV A, B, C1, C2, D; patient 2, CDC IV A, C2, D; patient 3, CDC IV A, C1, C2, D). All patients belonged to the risk group of homosexuals and were treated with 3′-azido-3′-deoxythymidine (AZT). When KS biopsy specimens were obtained, no KS-specific medical treatment was applied.

Tissue Samples. At least two samples of KS skin lesions were obtained from each patient by therapeutic surgery. Immediately after removal, these biopsy samples were fixed in 4% paraformaldehyde. Dehydration and embedding were carried out as described (32).

Vector Constructions. The transcription vectors pBV-sis1, pBRT7-A, and pT7ENV-19A were used for synthesis of RNA hybridization probes specific for PDGF-A, PDGF β receptor, and HIV-1, respectively. For construction of these vectors,

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Abbreviations: PDGF, platelet-derived growth factor; KS, Kaposi sarcoma; HIV-1, human immunodeficiency virus type 1.

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a 932-base-pair (bp) fragment spanning a region from exon 1 to exon 6 of v-sis (pBV-sis1), a 1161-bp *Hin*cII fragment coding for part of the mouse cDNA of PDGF β receptor (33) (pBRT7-A), and a 5100-bp *Pst* I fragment coding for the polymerase and envelope genes of HIV-1 isolate WMJ-2 (34) (pT7ENV-19A) were inserted into the multiple cloning site of pBluescript II SK(−) (Stratagene). The transcription vectors pBPDGF-A3′ (29), pPDGFrec-A (35), and pAL41 (36) were used for PDGF-A-, PDGF α receptor-, and β-actin-specific probes. Identity of the inserted sequences was verified by sequence analysis (data not shown). Transcription vectors used for synthesis of PDGF- and PDGF receptor-specific probes are presented in Fig. 1. Their hybridization specificity was examined by Northern blotting using as probes the respective fragments labeled by nick-translation (Fig. 1).

***In Situ* Hybridization.** Synthesis of ^{35}S-labeled complementary RNA probes and *in situ* hybridization were carried out (32) under high-stringency conditions. The RNA probe (specific activity, 10^9 cpm/μg) was applied to tissue sections at a final concentration of 50,000 cpm/μl in 50% (vol/vol) deionized formamide/0.3 M NaCl/20 mM Tris·HCl, pH 7.4/5 mM EDTA/10 mM sodium phosphate, pH 8/10 mM dithiothreitol/10% (wt/vol) dextran sulfate/1× Denhardt's solution containing total yeast RNA (50 μg/ml). After hybridization at

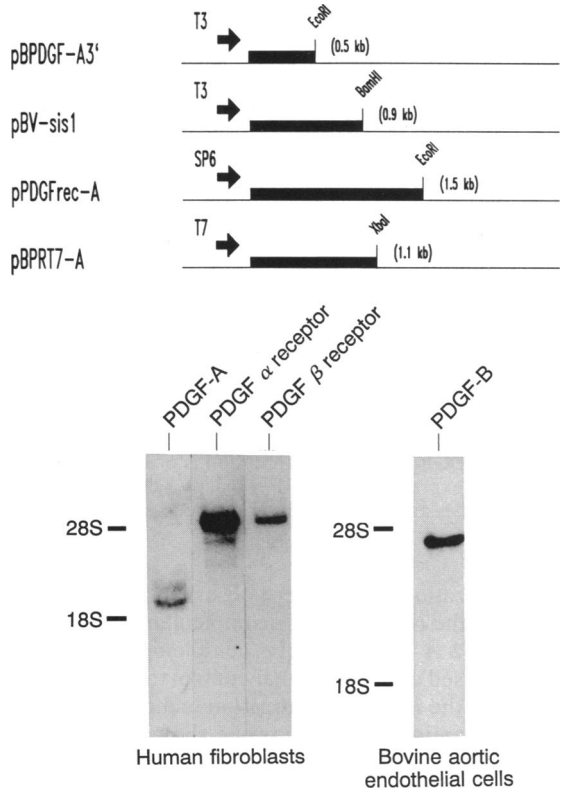

FIG. 1. Specificity of hybridization probes. (*Upper*) Sequences coding for PDGF-A, PDGF-B, PDGF α receptor, and PDGF β receptor were isolated from vectors pBPDGF-A3′, pBV-sis1, pPDGFrec-A, and pBPRT7-A, respectively. Restriction sites used for linearization of the template and the bacteriophage RNA polymerase (T3, SP6, or T7) used for synthesis of antisense hybridization probes for *in situ* hybridization are indicated. kb, Kilobase(s). (*Lower*) For Northern blotting isolated probes were radiolabeled by nick-translation and hybridized to 1.5 μg of poly(A)-enriched RNA, isolated from either human fibroblasts or bovine aortic endothelial cells. Each probe hybridized specifically to the corresponding mRNA: PDGF-A [2.8, 2.3, and 1.9 kb (37)]; PDGF-B [4.2 kb (37)]; PDGF α receptor [6.5 kb (38)]; PDGF β receptor [5.3 kb (38)]. Due to the conditions used for agarose gel electrophoresis, the 6.5-kb band and the 5.3-kb band of the PDGF receptor RNA could not be separated. Positions of 28S and 18S rRNA are indicated.

50°C for 16 hr, coverslips were floated off in 5× SSC (1× SSC is 0.15 M NaCl/15 mM sodium citrate, pH 7)/10 mM dithiothreitol at 50°C. Subsequently, tissue was subjected to a stringent washing at 60°C in 50% formamide/2× SSC/0.1 M dithiothreitol. After photographic development, slides were fixed and stained with hematoxylin and eosin. For each hybridization, control experiments were carried out on parallel sections under identical conditions. As a negative control, hybridization applying the radiolabeled sense strand of the respective probe was used. A positive control was performed using a probe specific for β-actin to demonstrate that all cells in the tissue could show hybridization.

Immunoperoxidase Staining. An ABC kit (Immunotech, Luminy, France) was used according to the supplier's conditions. For detection of respective antigens, the following dilutions of rabbit immunoglobulins [in phosphate-buffered saline containing 1% (wt/vol) bovine serum albumin] were applied as primary antibodies: anti-human PDGF-B, 1:20; anti-von Willebrand factor, 1:650; anti-S100, 1:200; anti-fibronectin, 1:1200; anti-collagen IV, 1:500. Anti-human PDGF-B IgG was purchased from Oncogene Sciences (Manhasset, NY); anti-collagen IV antibody was from Laboserv (Giessen, F.R.G.); the other antibodies were obtained from Dakopatts (Hamburg, F.R.G.). In addition, a monoclonal anti-muscle antibody (1:15,000; Ortho Diagnostics) was used as a primary antibody. Biotinylated affinity-purified goat $F(ab')_2$ fragments of anti-mouse (for anti-muscle primary antibody) or anti-rabbit (for all other antibodies) IgG (Jackson ImmunoResearch; diluted 1:1000 in 1% bovine serum albumin) were used as secondary antibodies. After peroxidase reaction, sections were counterstained in Harris hematoxylin. In control experiments, the primary antibody was replaced with bovine serum albumin.

Alkaline Phosphatase/Anti-Alkaline Phosphatase (APAAP) Technique. Platelets were detected with a monoclonal anti-platelet glycoprotein IIIa primary antibody (Dakopatts; 1:100 dilution in 50 mM Tris, pH 7.6/0.15 M NaCl) by using the APAAP system (Dakopatts). In control experiments the primary antibody was omitted.

Cell Culture, RNA Preparation, and Northern Blot Analysis. These techniques were employed as described (29).

RESULTS

To assess the functional potential of PDGF in the pathogenesis of KS *in vivo*, we performed *in situ* hybridization on sections of paraffin-embedded skin biopsy samples of three KS patients suffering from AIDS. Strand-specific *in vitro* transcribed RNA probes, specific for the different isoforms of PDGF and their receptors, were used. Transcription vectors for synthesis of PDGF- and PDGF receptor-specific probes are presented in Fig. 1 (see also *Materials and Methods*).

The expression of PDGF-A in the tumor was mostly restricted to cells surrounding vascular slits (Fig. 2*A*, arrow). In the text below these PDGF-positive cells will be termed KS-p cells. They differed from the characteristic spindle cells of KS with respect to their small, spherical nuclei with evenly distributed chromatin and inconspicuous nucleoli. In immunohistochemistry experiments using cell type-specific antibodies directed against von Willebrand factor, smooth muscle cells, nerve cell epitopes, fibronectin, or collagen IV, these cells did not show a clear staining in comparison to the respective positive controls (data not shown). In *in situ* hybridization with the PDGF-A-specific probe only a small fraction of spindle cells with typical elongated nuclei (KS-s cells) showed a signal, which was slightly higher than background. Fibroblasts in healthy regions of the skin and epithelial cells did not show a signal.

PDGF-B expression was restricted to the same cell population that expressed PDGF-A (Fig. 2*B*). The most prominent

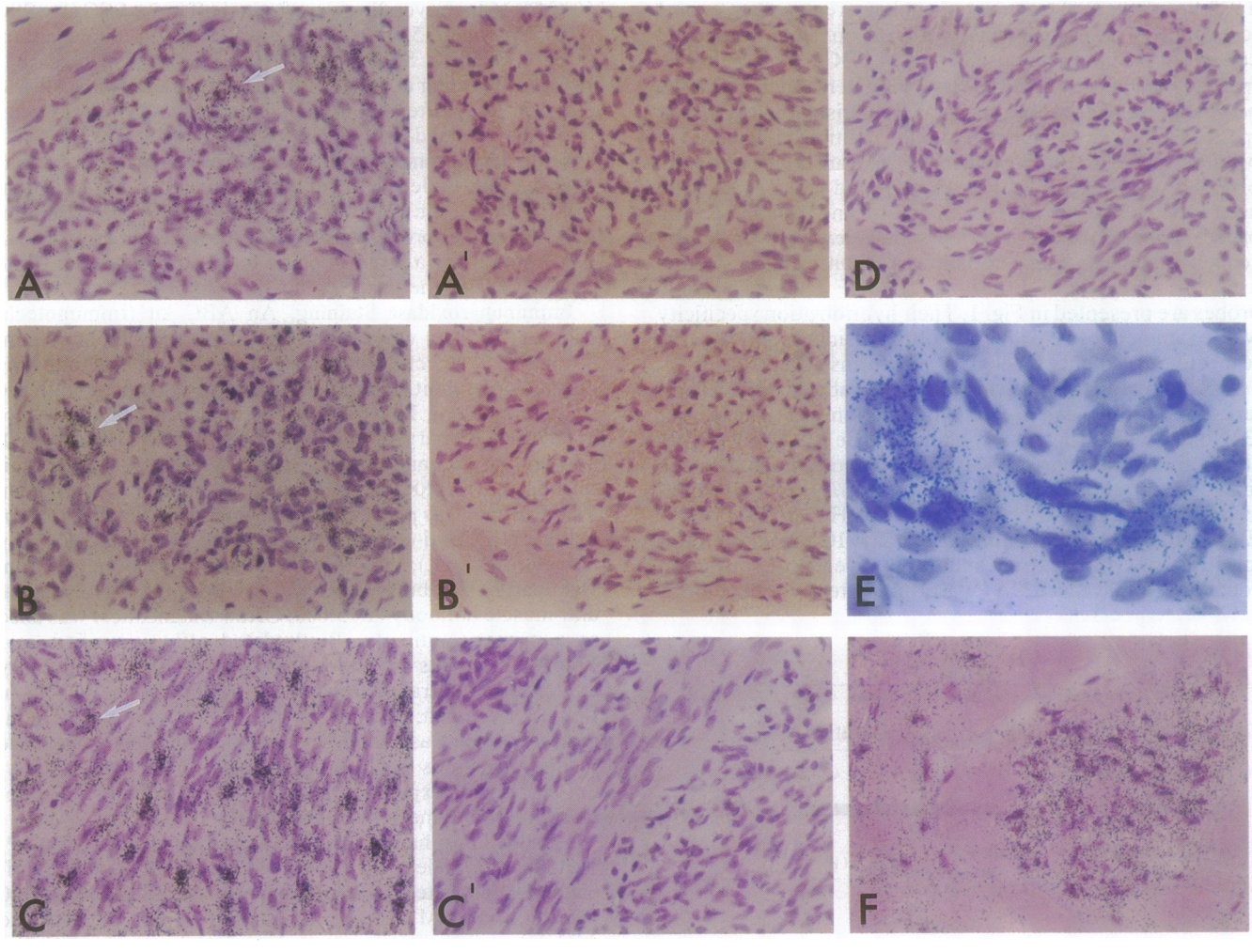

FIG. 2. *In situ* hybridization using ^{35}S-labeled PDGF- and PDGF receptor-specific antisense RNA probes on KS tissue sections. Hybridization with the respective sense strand revealed no background staining (*A'–C'*). PDGF-A expression is mostly restricted to cells grouped in whorls and surrounding vascular slits (KS-p cells, arrow). Only a few spindle cells (KS-s cells) express PDGF-A (*A*; negative control, *A'*). PDGF-B shows a similar expression pattern as PDGF-A. Cells surrounding vascular slits reveal the strongest hybridization signal (arrow) (*B* and *B'*). More detailed view of PDGF-B expression is seen at higher magnification (*E*). PDGF β-receptor expression is prominent in the KS-s cells and is also observed in KS-p cells surrounding vascular slits (arrow) (*C* and *C'*). No expression of PDGF α receptor can be detected (*D*). Hybridization with an antisense probe specific for β-actin demonstrates that the RNA probes are capable of hybridizing to all cells in the tissue section (*F*). (×240; except for *E*, ×600.)

signals were produced by cells positioned in whorls around vascular slits (arrow). The signal intensity was slightly higher compared with PDGF-A at equal exposure times (2 weeks). A more detailed view at higher magnification demonstrated more clearly that PDGF-B was predominantly expressed from KS-p cells surrounding vascular slits (Fig. 2*E*). Hybridization with a probe specific for the PDGF α receptor did not give any signal on KS-s, KS-p, and endothelial cells in the tumor (Fig. 2*D*). This was also true for a prolonged exposure up to 8 weeks (data not shown). Only some fibroblasts in the surrounding tissue showed a hybridization signal. In contrast, PDGF β receptor was highly expressed in all KS-s cells of the tumor (Fig. 2*C*). KS-p cells that stained positive for PDGF-A and PDGF-B also expressed PDGF β receptor (arrow). Compared with KS-p cells, the expression of the receptor in KS-s cells was slightly increased. Only a few fibroblasts in healthy regions of the skin showed a low expression of the PDGF β receptor, and epithelial cells did not express mRNA encoding this receptor form at all (data not shown). The latter observation is in agreement with data presented by Antoniades *et al.* (10), which were obtained by *in situ* hybridization of skin sections of swine.

No hybridization signals were observed in parallel experiments using the respective noncomplementary, sense RNA probes (Fig. 2 *A'–C'*). A probe complementary to β-actin mRNA was used as an additional control to demonstrate the specificity of the *in situ* hybridization technique. Hybridization with the antisense β-actin probe showed that mRNA of all cells in the tissue could be hybridized (Fig. 2*F*). Expression patterns of PDGF and PDGF receptor, presented above, suggested the existence of two KS cell types in the tumor (KS-s and KS-p cells), which might be stimulated by paracrine and autocrine mechanisms of PDGF action. Especially PDGF-B might be important for KS cell stimulation because only PDGF β receptor, which does not bind PDGF-A (3, 4), is expressed in the lesion. To determine whether PDGF-B mRNA detected in KS-p cells was also translated, sections of KS were stained with anti-human PDGF-B IgG. In accord with the results obtained from *in situ* hybridization, intense staining was observed in KS-p cells present in perivascular areas (Fig. 3*A*, arrow). KS-s cells in the tumor and fibroblasts in healthy regions of skin did not show any staining (Fig. 3*A*). No staining occurred when the anti-PDGF-B antibody was omitted (Fig. 3*B*).

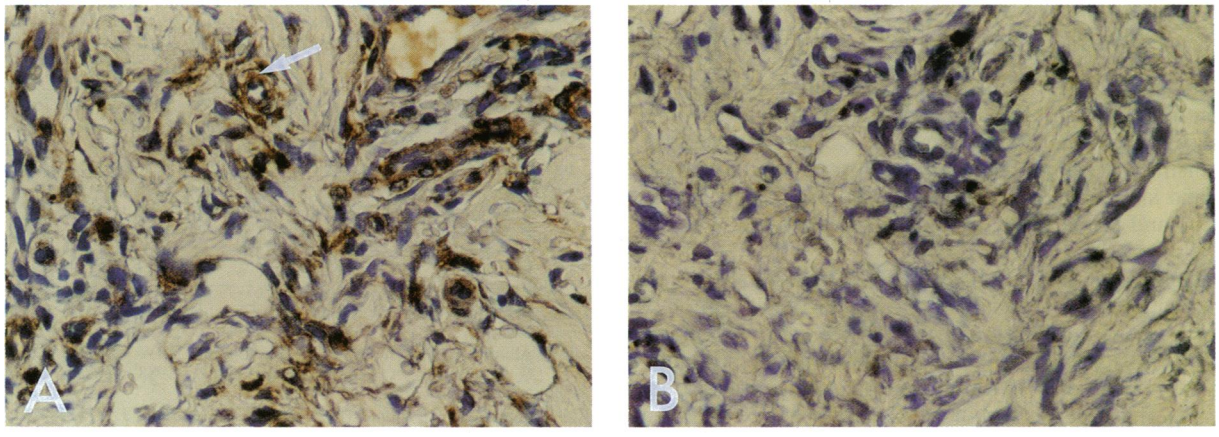

FIG. 3. Immunostaining for PDGF-B protein in KS tissue sections. Immunoperoxidase staining was carried out as described in *Materials and Methods*. Afterwards, sections were slightly counterstained with hematoxylin. Bright-field photographs are shown. (*A*) Immunostaining of PDGF-B protein is found mostly in KS-p cells surrounding vascular slits (arrow). KS-s cells and fibroblasts in healthy regions of the skin show no expression of PDGF-B. (*B*) Control staining under identical conditions as used in *A* but without anti-PDGF-B antibody. (×330.)

Paracrine effects of PDGF might be hampered because PDGF-B is not effectively secreted from living cells (39). Still, platelets might be an alternative reservoir to supply PDGF-B protein. Therefore, we investigated the presence of platelets in KS by using an antibody specific for platelet glycoprotein IIIa. Platelets could be detected in capillaries and vascular slits and in interstitium close to vascular slits (data not shown). No staining occurred in a control experiment where the anti-platelet antibody was omitted (data not shown).

To evaluate the direct influence of HIV-1 on pathogenesis of KS, we hybridized a probe specific for HIV-1 to sections of AIDS-associated KS. No signals were obtained from KS-s and KS-p cells (Fig. 4, lower part). Prominent signals occurred over a small number of cells in the epidermal layer most likely representing Langerhans cells (Fig. 4).

DISCUSSION

In situ hybridization of KS sections demonstrated simultaneous expression of PDGF-A, PDGF-B, and PDGF β receptor in cells grouped in whorls and surrounding vascular slits (KS-p cells). These cells were characterized morphologically by spherical nuclei, evenly distributed chromatin, and inconspicuous nucleoli. The majority of KS cells (KS-s cells) were characterized by spindle-like morphology and elongated nuclei. KS-s cells expressed PDGF β receptor only.

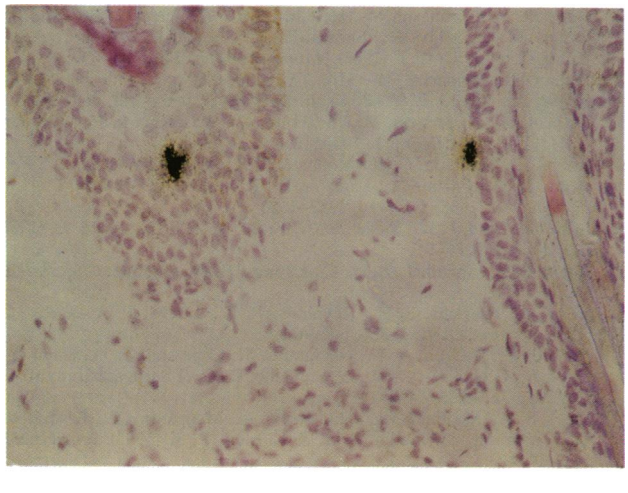

FIG. 4. Hybridization for HIV-1 in KS. Virus sequences were not detected in the spindle cells of KS by hybridization with an ^{35}S-radiolabeled antisense probe specific for HIV-1 (lower part). Strong signals were obtained from cells in the epidermal layer. These cells might represent HIV-1-infected Langerhans cells. (×230.)

Simultaneous expression of PDGF and PDGF receptors is characteristic for aortic smooth muscle cells and neoplastic cells (2, 40). Microvascular endothelial cells also express PDGF-A and -B as well as PDGF β receptors and respond to PDGF mitogenically (8, 9). In contrast, expression of PDGF receptors and lack of PDGF expression, which was found in KS-s cells, are characteristic for fibroblasts (1). The only exceptions known so far are fibroblasts in very early stages of wound healing, which express both PDGF-B and PDGF β receptors (10). The expression pattern of PDGF and PDGF receptor in sections of KS suggests that the tumor cell population is constituted of two cell types originating from different ancestor cells: endothelium-derived KS-p cells, with round nuclei expressing PDGF-A, PDGF-B, and β receptor, and spindle-shaped KS-s cells, with elongated nuclei and originating from fibroblasts expressing only PDGF β receptor. This hypothesis is supported by immunohistochemical staining results of others who found expression of an endothelial cell marker, von Willebrand factor, primarily in cells surrounding vascular slits (13, 15, 41, 42). Staining of KS sections for von Willebrand factor in our laboratory did not give clear results with respect to KS-p cells. Very few of these cells were slightly positive at best, whereas endothelial cells were definitely positive and KS-s cells were negative. Unclear or negative staining for other cell markers (fibronectin, collagen IV, smooth muscle cell, and nerve cell epitopes) did not provide contradictory or additional information about the origin of KS-p and KS-s cells.

We suggest that PDGF may act in the development of KS in a concerted autocrine and paracrine manner. In KS lesions, autocrine mechanisms of growth stimulation are most likely to be constituted by KS-p cells expressing both PDGF and PDGF receptors. We observed a strong staining for PDGF-B protein in these cells, indicating that high amounts of PDGF-B were synthesized. Upon release PDGF-B may also promote the growth of nearby KS-s cells expressing PDGF receptors (paracrine stimulation). Nevertheless, PDGF-B release from intact cells may be hindered by the presence of a cell retention signal in the PDGF-B polypeptide (39). Therefore, we investigated the possibility of a release from platelets, the major source of PDGF in serum. Using an antibody directed against a platelet epitope, we found platelet-specific staining along the inner wall of capillaries and vascular slits, as well as in the interstitium close to vascular slits (data not shown). We thus conclude that PDGF

released from degranulating platelets may contribute to a high local PDGF content and possibly constitute the majority of the total paracrine PDGF activity in KS.

PDGF is not only a potent mitogen but also a potent chemoattractant for fibroblasts and smooth muscle cells (1). This may suggest that PDGF works in KS through multiple mechanisms: (*i*) via cell migration by attracting cells (fibroblasts and smooth muscle cells) into the tumor and (*ii*) via autocrine and primarily paracrine growth stimulation of KS cells.

The high incidence of KS in HIV-1-infected individuals, together with the finding that HIV-1 induces the formation of KS-like lesions in transgenic mice (43), led us to investigate HIV-1 expression in KS by *in situ* hybridization. We found strong expression only in a few epidermal cells, most probably Langerhans cells. Tumor cells were negative. We can exclude that KS cells with a productive infection of HIV-1 remain undetected by our *in situ* hybridization. The detection of low-abundance growth factor mRNA (Fig. 2) demonstrates the high sensitivity of our technique.

The low number of HIV-1-positive cells might be attributed to 3′-azido-3′-deoxythymidine treatment of the KS patients. Nevertheless, at the time biopsy samples were obtained, development of KS lesions was still in progress. Therefore, productive infection of KS spindle cells by HIV-1 can be excluded as an important factor for KS tumor progression. However, HIV-1 might activate KS cell growth *in vivo* by a positive systemic or an indirect local effect, mediated by factors such as oncostatin M (44, 45) and the Tat protein (24). Both factors are synthesized by lymphatic cells subsequently to HIV-1 infection and stimulate proliferation of KS-derived cells *in vitro* (24, 44, 45). *In vivo*, the concerted action of oncostatin M, Tat protein, and PDGF might be important for initiation and progression of KS.

We thank the organizers of the European Molecular Biology Organization course "*In situ* hybridization on the mouse" (1990), especially M. Buckingham, M. Brahic, and G. Lyons (Institut Pasteur, Paris) and D. Sasson (Boston University School of Medicine), for their outstanding performance in teaching the method of *in situ* hybridization. We thank S. Werner (University of California, San Francisco), C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala), A. Ullrich (Max-Planck-Institut für Biochemie, Martinsried, F.R.G.), and H. Wolf (Max von Pettenkofer Institut, Munich) for providing vector constructions (pBPDGF-A3′, pPDGFrec-A, pmPDGFrec-HincII, and WMJ2). Special thanks go to W. Risau for critically reading the manuscript. This work was supported by Bundesministerium für Forschung und Technologie (II-033-87 and BGA-III-002-89).

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