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# Tumor–host interaction mediates the regression of BK virus-induced vascular tumors in mice: involvement of transforming growth factor-β1

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**Several sexually transmitted viruses have been associated with the development of Kaposi's sarcoma (KS), a highly vascularized multi-focal neoplasm, characterized by the presence of spindle-shaped and endothelial cells, fibroblasts and macrophages. As BK virus (BKV) sequences were found in 100% of primary KS and 75% of KS cell lines, we established an experimental model to test whether BKV may be involved in the pathogenesis of KS. For this purpose, we transformed primary and spontaneously immortalized murine endothelial cells with BKV or with a plasmid containing BKV early region, which encodes BKV T antigen. Murine endothelial cells lost endothelial markers after transformation by BKV and, when inoculated s.c. in nude mice, induced tumors which regressed 7–30 days after onset, whereas spontaneously immortalized murine endothelial MHE cells induced progressing tumors, which brought the animals to death. Histologic examination showed an initial formation of vessels around the tumors, followed by the appearance of a dense population of fibroblasts and mononuclear cells in the peritumoral tissue. Subsequently, tumors appeared to be infiltrated by mononuclear cells and surrounded by a thick fibrous wall with scattered fibroblasts and without vessels. Areas of necrosis developed in the tumor mass and finally the neoplastic tissue completely degenerated. The medium conditioned by BKV-transformed cells induced proliferation and migration of human fibroblasts and NIH3T3 cells. These effects were inhibited by an anti-transforming growth factor-β1 (TGF-β1) antibody. Northern blot analysis revealed that BKV-transformed cells express a greater amount of TGF-β1 RNA than normal murine endothelial cells. Besides, TGF-β1 was not expressed in progressing tumors induced by spontaneously immortalized endothelial MHE cells, whereas it was highly expressed during the regression of tumors induced by BKV-transformed MHE and primary endothelial cells. Over-expression of TGF-β1 may be responsible for the mononuclear cell infiltration, inhibition of angiogenesis and formation of the fibrotic wall around tumors, inducing tumor regression through tumor cell necrosis and nutritional starvation. These results prompt us to test whether production of TGF-β1 is associated with spontaneous KS regression in human patients. In this case, KS regression could be induced or accelerated by any means that enhances TGF-β1 production at the tumor site.**

**Abbreviations:** bFGF, basic fibroblast growth factor; BKV, BK virus; CM, conditioned medium; DMEM, Dulbecco's modified Eagle's minimal essential medium; FCS, fetal calf serum; KS, Kaposi's sarcoma; IL-6, interleukin 6; PA, plasminogen activator; TAg, BKV T antigen; TGF-β1, transforming growth factor-β1.

## Introduction

Kaposi's sarcoma (KS) is a highly vascularized multi-focal neoplasm, distinguished in four epidemiologic forms: classic, endemic, iatrogenic and AIDS-associated. Lesions of all forms of KS are characterized by the presence of spindle-shaped and endothelial cells, fibroblasts, macrophages and an intense inflammatory infiltrate (1). Besides, due to the immunoreactive origin of KS, it has been suggested that a sexually transmitted agent may participate in the etiopathogenesis of this tumor (2). Several sexually transmitted viruses, such as cytomegalovirus (3,4), hepatitis B virus (5), papillomavirus (6,7), retroviruses (8), BK virus (BKV) (9), human herpesvirus (HHV) 6 (10) and HHV 8 or KS herpesvirus (11), have been associated with the development of KS by biological, morphological and molecular evidence. Although more than one virus may be causally related to KS, either in the early or in the late phase of tumor development, recent studies have supported a role for HHV 8 as the principal etiologic agent of KS (12).

BKV is a human papovavirus ubiquitous in the human population and with a worldwide distribution (13). After the primary infection, which is often asymptomatic, the virus persists in a latent state. However, BKV infection is reactivated in immunosuppressed hosts and sometimes in healthy individuals. BKV transforms rodent and human cells (14) and is highly oncogenic when inoculated into newborn hamsters (15,16). Moreover, BKV infection has been associated with the development of human tumors (17). BKV T antigen (TAg), a nuclear transforming protein homologous to SV40 TAg, binds the products of *p53* and *RB* tumor-suppressor genes inactivating their function (18,19) and induces mutations as well as structural and chromosomal aberrations in host cells (20,21). BKV TAg also cooperates with c-*ras* for tumor induction in hamsters and for transformation of rodent and human cells (22). These results support the notion that BKV may play a role in the multi-step process of carcinogenesis. Furthermore, the presence of BKV sequences in 100% of primary KS and 75% of KS cell lines (23) suggests that BKV may be involved in the pathogenesis of KS.

Several experimental results show that murine endothelial cells, transformed by SV40 or polyoma virus—two papovaviruses similar to BKV—exhibit features of KS-derived cells. In fact, they secrete cytokines such as interleukin 6 (IL-6),

monocyte chemoattractant protein and granulocyte-macrophage colony stimulating factor, which behave as autocrine and paracrine factors, and induce hemangiomas and KS-like tumors in nude mice by recruitment of host endothelial cells (24–26). This murine model recapitulates some pathogenetic events occurring in KS, where spindle-shaped cells represent the core of the lesion and elicit an unbalanced network of soluble mediators, which seem to be essential for mesenchymal cell recruitment and lesion growth (12). The molecular and cellular basis of cell recruitment, contributing to the development of human tumors, including KS, as well as the ontogenesis of human hemangiomas are largely unknown (27).

In order to test a possible contribution by BKV to the development of KS, in the present study we have analyzed whether BKV induces endothelial cell proliferation and angiogenesis. To this purpose, murine endothelial cells were transformed with BKV or with a plasmid containing BKV early region. The behavior of BKV-transformed cells was studied *in vitro* and after injection in nude mice.

## Materials and methods

*Cell cultures*

Primary endothelial cells were prepared from brains of 13-day-old DBA/2 male mice (Charles River, Como, Italy). The tissue was minced and digested with 0.25% collagenase A from *Clostridium histolyticum* (Boehringer Mannheim, Mannheim, Germany) for 3 h at 37°C. The suspension was subsequently filtered through 100 and 40 mesh screen grinder, respectively. Cells were plated on 6-well plates (Falcon, Becton Dickinson, San Jose, CA) coated with rat collagen I (10 μg/ml; Sigma Chemical Co., St Louis, MO) and human fibronectin (10 μg/ml; Sigma), incubated for 1 h and washed four times with cold phosphate-buffered saline (PBS). Cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma) supplemented with 20% fetal calf serum (FCS) (Irvine, Santa Ana, CA), endothelial cell growth supplement (100 μg/ml; Sigma) and porcine heparin (100 μg/ml; Sigma). Every day the cultures were extensively washed with cold PBS. After a few days, endothelial cells were purified using magnetic beads (Dynal A.S., Oslo, Norway) conjugated with a monoclonal rat anti-mouse CD31 antibody (28) (a gift of Dr A.Vecchi, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy) according to the manufacturer's instructions. Spontaneously immortalized murine Balb/c MHE cells (29), NIH3T3 cells (American Type Cellular Collection, Rockville, MD) and human dermal fibroblasts (Clonetics, BioWhittaker Europe, Verviers, Belgium) were grown in DMEM with 10% FCS at 37°C in a humidified atmosphere containing 5% $CO_2$.

*Detection of endothelial cell-specific markers*

Two techniques were used to detect endothelial cell-specific markers, immunophenotyping and western blot.

*Immunophenotyping*. Confluent cell monolayers were washed twice with PBS and fixed for 5 min at 0°C with 3% formaldehyde/2% sucrose, pH 7.6. When required, cells were additionally permeabilized with Triton X-100 buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) for 3 min at 0°C (30). Cells were stained with the following monoclonal antibodies: rat anti-mouse CD31, rat anti-mouse von Willebrand factor (vWF; Roche Diagnostics, Monza, Italy), rat anti-mouse vascular endothelial growth factor receptor-2 (VEGFR-2; Chemicon, Temecula, CA), rat anti-mouse vimentin or cytocheratin (Sigma) and with a rabbit polyclonal antibody against anti-nitric oxide synthase (NOS) type III (BD Biosciences Pharmingen, San Diego, CA) or anti-VE-cadherin (31) (a gift from Dr E. Dejana, Mario Negri Institute, Milan, Italy) for 30 min at 37°C. After extensive washing, cells were stained with a goat anti-rat or anti-rabbit IgG labeled with fluorescein (Sigma). Coverslips were mounted on microscope glasses with 20% Moviol 4-88 (Hoechst, Frankfurt, Germany) and observed with a Zeiss Axiophot microscope. The uptake of acetylated-LDL was evaluated as described previously (25).

*Western blot*. Cells ($5 \times 10^6$) were washed and lysed at 4°C in 25 mM HEPES buffer pH 7.6 containing 150 mM NaCl, 1% Triton X-100, protease and phosphatase inhibitors (pepstatin, 50 ng/ml; leupeptin, 50 ng/ml; aprotinin, 10 μg/ml and 1 mM PMSF). Cell lysate (1 mg) was immunoprecipitated with a monoclonal rat antibody anti-mouse VE-cadherin (10 mg; BD Biosciences Pharmingen) or with a monoclonal rat antibody anti-mouse CD31 (5 mg; BD Biosciences Pharmingen) or with a goat polyclonal antibody against actin (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins solubilized from beads were separated by electrophoresis in 10% polyacrylamide gels, transferred to Immobilon-P sheets (Millipore, Bedford, MA) and probed with the following polyclonal antibodies: goat anti-rat for VE-cadherin (Santa Cruz) and CD31 (Santa Cruz), and rabbit anti-goat for actin. The enhanced chemiluminescence technique (NEN, Perkin Elmer Cetus, Norwalk, CT) was used for the detection of bands.

*BKV plasmid and virus*

The vector pRP*neo*-c, containing BKV early region and origin of replication and the *neo* gene as the selectable marker, was described previously (32). BKV stocks were produced in VERO cells as described (33). Virus was titrated by haemagglutination of type 0 human erythrocytes (34) and by the fluorescent antibody (FA) focus-forming assay in human embryonic fibroblasts (35).

*Transformation of murine endothelial cells with BKV and pRPneo-c*

Subconfluent mouse primary endothelial cells were infected with BKV at 100 FA focus-forming units per cell. Two months after infection cells began to detach from the monolayer and small colonies started to grow up. They were isolated, propagated and expanded into continuous cell cultures. Transfection with pRP*neo*-c was performed on second passage subconfluent endothelial cells and on the MHE cell line. Cells ($1 \times 10^6$) were transfected with 5 μg of pRP*neo*-c by the calcium-phosphate precipitation technique (36) and cultured in medium containing G418 (200 μg/ml; Sigma). After 3–4 weeks of selection, the G418-resistant colonies were isolated and expanded. Transformed cells were grown and maintained in DMEM with 10% FCS.

*Analysis of BKV TAg expression*

BKV TAg was detected by indirect immunofluorescence. Subconfluent cell monolayers were fixed for 10 min in cold acetone, incubated for 30 min at 37°C with hamster serum to BKV TAg, washed three times in PBS, and incubated for 30 min at 37°C with fluorescein-conjugated rabbit anti-hamster IgGs (Antibodies Incorporated, Davis, CA). After extensive washing in PBS, the preparations were mounted in buffered glycerol and observed with a fluorescence microscope (Olympus Optical Co., Hamburg, Germany).

*Southern and northern blot hybridization*

Total cellular DNA was prepared from cultured cells as described previously (35). To detect pRP*neo*-c DNA in transformed cells, genomic DNA was digested with *Eco*RI or *Bam*HI restriction enzymes, which cut pRP*neo*-c DNA once and twice, respectively. DNA fragments were separated onto 0.8% agarose gels, blotted to nitrocellulose membranes according to Southern (37) and hybrized to a $^{32}$P-labeled pRP*neo*-c DNA probe. The dried membranes were exposed to a Kodak X-Omat SO-282 film at −70°C with an intensifying screen. To detect transforming growth factor (TGF)-β1 transcripts, total cytoplasmic RNA was extracted from cell lines by standard procedures (37). Northern blot hybridization was performed as described previously (38), using as probe the 1.7 kb *Bam*HI fragment of the murine TGF-β1 cDNA. pRP*neo*-c and TGF-β1 DNA probes were labeled with [α-$^{32}$P]dCTP (3000 Ci/mM; Amersham-Biotech-Pharmacia, Buckinghamshire, UK) by nick-translation (39) at a specific activity of $2–4 \times 10^8$ c.p.m./μg. TGF-β1 transcripts were quantified by densitometric analysis using the GS250 Molecular Imager Reader (Bio-Rad Laboratories, Hercules, CA).

*Tumorigenicity assay, histological and immunohistochemical procedures*

Cells were dispersed with trypsin–EDTA, washed in DMEM plus 10% FCS and re-suspended in DMEM without serum. Four-week-old Balb/c nude mice were inoculated subcutaneously with $1–5 \times 10^6$ cells. Mice were examined daily for appearance of tumors and the size of tumors were measured with a caliper. Six days after tumor appearance, some mice were killed and tumors removed for histological examination. Tumors were fixed in 70% ethanol, embedded in paraffin and stained by hematoxylin and eosin (H&E) according to standard histological procedures.

The avidin–biotin–peroxidase complex (ABC) technique was used for the assay of TGF-β1 expression in paraformaldehyde-fixed and paraffin-embedded tissue sections. The samples were de-waxed for 12 min at room temperature with Histolemon (Carlo Erba Reagenti, Milan, Italy), washed in absolute ethanol, then incubated for 30 min at room temperature with 1% hydrogen peroxide in methanol. After dehydration in ethanol, slides were rinsed in distilled water and PBS, and pretreated for 12 min at room temperature with 0.1% trypsin in PBS. Aspecific binding was blocked by 1 h incubation at room temperature in 10% normal goat serum in PBS containing 10% BSA, then slides were incubated overnight at 4°C with primary rabbit anti-TGF-β1

antibody (Santa Cruz Biotechnology). After rinsing in PBS, they were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature, followed by treatment with ABC (Vector Laboratories) according to manufacturer's instructions. Peroxidase activity was visualized by 10 min incubation with diaminobenzidine (Vector Laboratories) and then slides were counterstained with hematoxylin and mounted in lemonvitrex (Carlo Erba Reagenti).

*Proliferation and migration assays*

Conditioned medium (CM), obtained from culture supernatants of normal, BKV- and pRP*neo*-c-transformed endothelial cells, maintained for 48 h in DMEM without FCS, was centrifuged at 1500 r.p.m. for 10 min to remove cellular debris, filtered through 0.22 μm Millipore filters, diluted with DMEM and added to human dermal fibroblast cultures. In a group of experiments, CM, diluted 1:2 or human recombinant TGF-β1 (5 ng/ml; R&D Systems, Abingdon, UK) were incubated overnight at 4°C with an affinity purified rabbit polyclonal anti-TGF-β1 antibody (R&D Systems) or with normal rabbit IgG as a control (0.2 μg/ml; Sigma). Human fibroblasts were seeded in 96-well plates (Falcon, Becton Dickinson) ($1 \times 10^3$ cells per well) in DMEM containing 10% FCS. After 24 h cells were washed and incubated for 8 days with CM of normal endothelial or BKV-transformed cells or with human recombinant TGF-β1. Fibroblast number was estimated after staining with crystal violet by a colorimetric assay described previously (40). The stained cells were solubilized with acetic acid (10%) and the color intensity of the lysate was measured at 595 nm in a Microplate Reader (model 3550; Bio-Rad Laboratories). A calibration curve was set up with a known number of cells. Proportionality between absorbance and cell counts exists up to $5 \times 10^4$ cells.

Cell migration was evaluated with a chemotaxis microchamber assay as described previously (38) using a 5 μm pore size polycarbonate filter (Nucleopore, Pleasanton, CA). Twenty-seven milliliters of chemoattractant solution, represented by CM or human recombinant TGF-β1, were added to the lower wells of the chemotaxis chamber (Neuroprobe, Bethesda, MD), and $7.5 \times 10^4$ human fibroblasts or NIH3T3 cells were seeded in the upper wells. Incubation was carried out at 37°C in air with 5% $CO_2$ for 4 h. Filters were removed, fixed and stained with Diff-Quick (Baxter Spa, Rome, Italy) and cells from five high-power oil-immersion fields per preparation were counted.

*Statistical analysis*

All the experiments were performed in triplicate. One-way analysis of variance and the Student–Newman–Keuls test were used for the statistical analysis. Values of $P \leq 0.05$ were considered significant.

## Results

*Transformation of murine endothelial cells and characterization of early-passage transformed cells*

Mouse primary brain endothelial cells were either infected with BKV or transfected with pRP*neo*-c, whereas MHE cells were only transfected with pRP*neo*-c. Primary endothelial cells either infected (Brain-BK) or transfected (BBKI) and MHE transfected cells developed a large number of transformed foci, whereas normal primary brain endothelial cells became senescent; the time of appearance of the transformed foci was shorter in transfected cell cultures. All transformed clones grew to a greater saturation density and showed an altered morphology as compared with control cells (Figure 1A–C). Moreover, BKV TAg was expressed in ~100% of the nuclei of all transformed cell clones as detected by immunofluorescence (Figure 1D). Analysis of endothelial cell markers by immunophenotyping revealed that CD31, vWF, VE-cadherin, VEGFR-2 and NOS type III were expressed in all or in the great majority of primary brain cells, whereas their expression was markedly reduced or disappeared in transformed cells (Table I). Disappearance of CD31 and VE-cadherin in BKV-transformed endothelial cells was confirmed by western blot (Figure 2). On the other hand, the expression of vimentin persisted, confirming the mesenchymal origin of transformed cells (Table I). These results indicate that BKV transformation is associated with the loss of endothelial cell differentiation markers.

*Presence of pRPneo-c in transformed cells*

Eight clones of BBKI cells transformed by pRP*neo*-c were studied in detail for the presence and state of the recombinant DNA. Total cellular DNA was digested with *Bam*HI, which cuts the recombinant DNA at two sites, releasing two fragments of 8.6 and 2.6 kb, and analyzed by Southern blot

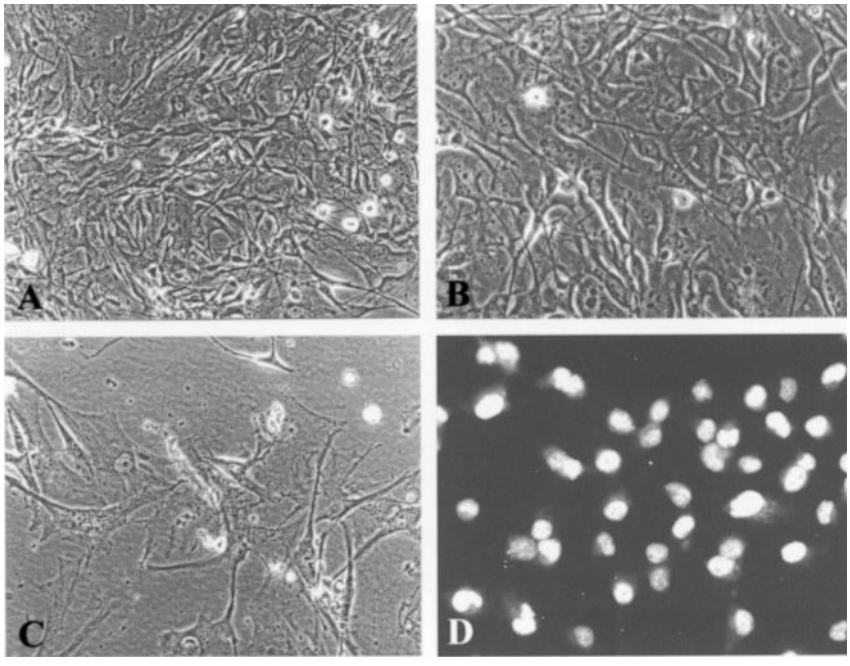


**Fig. 1.** Morphological analysis of primary brain endothelial cells transformed by pRP*neo*-c. One month after transfection with pRP*neo*-c, cells showed morphological alterations and grew to a greater saturation density, giving rise to continuous cell lines (**A** and **B**), whereas untransfected cells diplayed signs of senescence and detached from the monolayer (**C**). All BKV-transformed cells expressed BKV TAg (**D**). Original magnification ×100 (A–C). In (D) cells were reacted with a hamster serum to BKV TAg, then with fluorescein-conjugated rabbit anti-hamster IgG and observed with a UV miscroscope. Original magnification ×800.

**Table I.** Endothelial markers detected in primary and BKV-transformed murine cells

| Markers[a] | Primary endothelial cells[b] | BBKIcl6[c] | Brain-BKcl1[c] | Brain-BKcl5[c] | Brain-BKcl9[c] |
|---|---|---|---|---|---|
| CD31 | 90 ± 3 | 5 ± 2 | 7 ± 3 | 2 ± 1 | 0 |
| vWF | 51 ± 6 | 0 | 0 | 6 ± 2 | 0 |
| VE-cadherin | 100 | 4 ± 2 | 6 ± 3 | 10 ± 5 | 0 |
| NOS type III | 89 ± 4 | 45 ± 6 | 41 ± 13 | 27 ± 8 | 23 ± 7 |
| VEGFR-2 | 100 | 3 ± 2 | 9 ± 7 | 0 | 0 |
| Cytocheratin | 0 | 0 | 0 | 0 | 0 |
| Vimentin | 100 | 100 | 100 | 100 | 100 |

[a]All values of marker expression are shown as percentage of positive cells in five microscopic fields.
[b]Mean ± SD of three different coverslips seeded with cells at the second passage.
[c]Mean ± SD of three different coverslips seeded with cells at the third passage after the establishment of transformation. Similar results were obtained at subsequent culture passages.

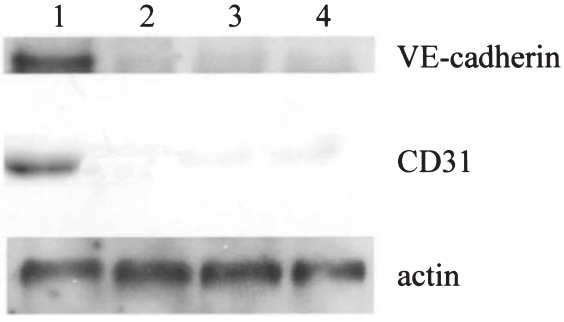


**Fig. 2.** Cells ($5 \times 10^6$) were lysed and proteins (1 mg) were immunoprecipitated by specific antibodies as described in Materials and methods. Immunoprecipitated proteins were separated by electrophoresis in 10% polyacrylamide gels and blotted with antibodies to VE-cadherin, CD31 or actin. Lane 1, primary endothelial cells; lane 2, BBKIcl6; lane 3, Brain-BKcl1; lane 4, Brain-BKcl5. This figure is representative of two experiments performed with the same results.

hybridization. All clones, except clone 7, showed two hybridization bands, suggesting a single insertion of a defective pRP*neo*-c molecule, which has lost a *Bam*HI site during integration into the host cell genome (Figure 3, lanes 1–6 and 8). In fact, if one copy of pRP*neo*-c DNA was integrated as an intact molecule into the host cell genome, after digestion with *Bam*HI one would expect to detect a band co-migrating with one of the two bands of the pRP*neo-c* control DNA (Figure 3, lane 9). Clone 7 showed four hybridization bands (Figure 3, lane 7), suggesting two single insertions of the transfected DNA. pRP*neo*-c DNA was rearranged also in clone 7, because no bands co-migrating with the digestion products of control pRP*neo*-c (Figure 3, lane 9) were detected. pRP*neo*-c rearrangements in transformed cell clones, however, should not affect BKV early region, because BKV TAg was expressed in the nuclei of all transformed cell clones (Figure 1D). Digestion of cellular DNA with *Eco*RI, which has one restriction site in pRP*neo*-c, confirmed the results obtained with *Bam*HI digestion (data not shown).

*Tumorigenicity of BKV- and pRPneo-c-transformed cells and histological features of tumors*

The tumorigenicity of BKV-transformed cell clones was investigated in nude mice. Four-week-old nude mice were

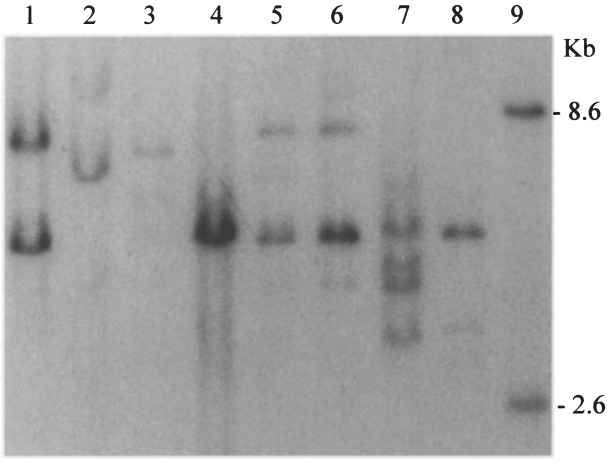


**Fig. 3.** Southern blot analysis of total cellular DNA extracted from pRP*neo*-c-transformed primary endothelial cells. DNA (20 μg/lane) was analyzed after cleavage with *Bam*HI. Lanes 1–8, BBKIcl1, cl2, cl3, cl4, cl5, cl6, cl7 and cl9; lane 9, pRP*neo*-c plasmid DNA (2.5 genome equivalents per cell) cut with *Bam*HI. *Bam*HI has two cleavage sites on pRP*neo*-c and produces two fragments of 8.6 and 2.6 kb.

**Table II.** Oncogenicity of BKV-transformed murine endothelial cells for nude mice

| Cell lines[a] | Mice with tumor/Mice inoculated | Tumor appearance (days after inoculation)[b] | Tumor regression[c,d] (days after appearance) |
|---|---|---|---|
| BBKIcl1 | 4/4 | 5 | 17.0 ± 3.4 |
| BBKIcl2 | 2/4 | 14 | 12.0 ± 0 |
| BBKIcl3 | 4/4 | 3 | 12.0 ± 5.1 |
| BBKIcl5 | 4/4 | 5 | 16.2 ± 8.1 |
| BBKIcl6 | 2/4 | 7 | 7.0 ± 0 |
| BBKIcl7 | 2/4 | 5 | 10.0 ± 5.6 |
| BBKIcl9 | 4/4 | 4 | 11.5 ± 2.8 |
| Brain-BKcl1 | 3/4 | 17 | 13.0 ± 0 |
| Brain-BKcl2 | 4/4 | 17 | 13.0 ± 0 |
| Brain-BKcl3 | 2/4 | 16 | 13.0 ± 0 |
| Brain-BKcl4 | 4/4 | 4 | 24.7 ± 25.5 |
| MHE control | 4/4 | 6 | 91.0 ± 69.2 |
| BK-MHEcl1 | 2/4 | 12 | 11.0 ± 0 |
| BK-MHEcl3 | 3/4 | 4 | 20.3 ± 10.6 |
| BK-MHEcl9 | 3/4 | 10.3 ± 4.2 | 30.3 ± 20.2 |

[a]Mice were inoculated with 1–5 × $10^6$ cells.
[b]Tumors appeared at the same day in each group of mice with the exception of tumors induced by BK-MHE cl9 cells. In this case SD was calculated.
[c]After tumor regression all mice survived, including two mice, inoculated with MHE control cells. The other two mice inoculated with MHE control cells developed tumors, which progressed and brought the animals to death.
[d]The regression time of tumors induced by BKV-transformed MHE and primary murine endothelial cells was significantly shorter than that of tumors induced by MHE control cells: $P < 0.0005$.

inoculated s.c. with 1–5 × $10^6$ cells per mouse. Shortly after inoculation of cells (3–17 days), animals developed s.c. tumors, classified as sarcomas. All tumors, however, completely regressed 7–30 days after appearance (Table II). In addition, none of the injected animals developed metastases, and they survived without resumption of tumor growth. All animals inoculated with the spontaneously immortalized MHE control cells developed tumors: contrary to tumors induced by BKV-transformed cells, in this case two tumors regressed in a long period of time (91 days) and two other tumors increased

in size, progressed and brought to death the mice (Table II). The regression time of tumors induced by BKV-transformed MHE and primary murine endothelial cells was significantly shorter than that of tumors induced by MHE control cells ($P < 0.0005$; Table II).

The development and regression of tumors was followed by histological examination. At the beginning, newly formed small vessels developed around the tumor (Figure 4A). After a short period of growth, tumors were surrounded by a conspicuous cell population, made up of spindle-shaped and mononuclear cells that started to invade the tumor mass (Figure 4B and C). The cell infiltration around the tumors later evolved to form a thick fibrous wall without vessels, tumor cells started to degenerate and necrotic areas developed within the tumor (Figure 4D). The final stages of tumor regression led to replacement of the neoplastic tissue by a fibroblast network and a mononuclear cell infiltrate (Figure 4E). In some cases, due to progression of necrosis, the whole tumor degenerated, leaving an empty cavity surrounded by the fibrous wall (Figure 4F). The histological aspect of tumors induced by spontaneously immortalized MHE control cells was different: the tumors were not surrounded by a fibrous wall, tumor cells proliferated and the tumor mass expanded (data not shown).

*Proliferation and migration of human fibroblasts and NIH3T3 cells in CM of BKV-transformed cells*

Since tumors were surrounded by a thick fibrous wall, we considered the possibility that BKV-transformed cells release fibrogenic factors that cause the proliferation of fibroblasts and

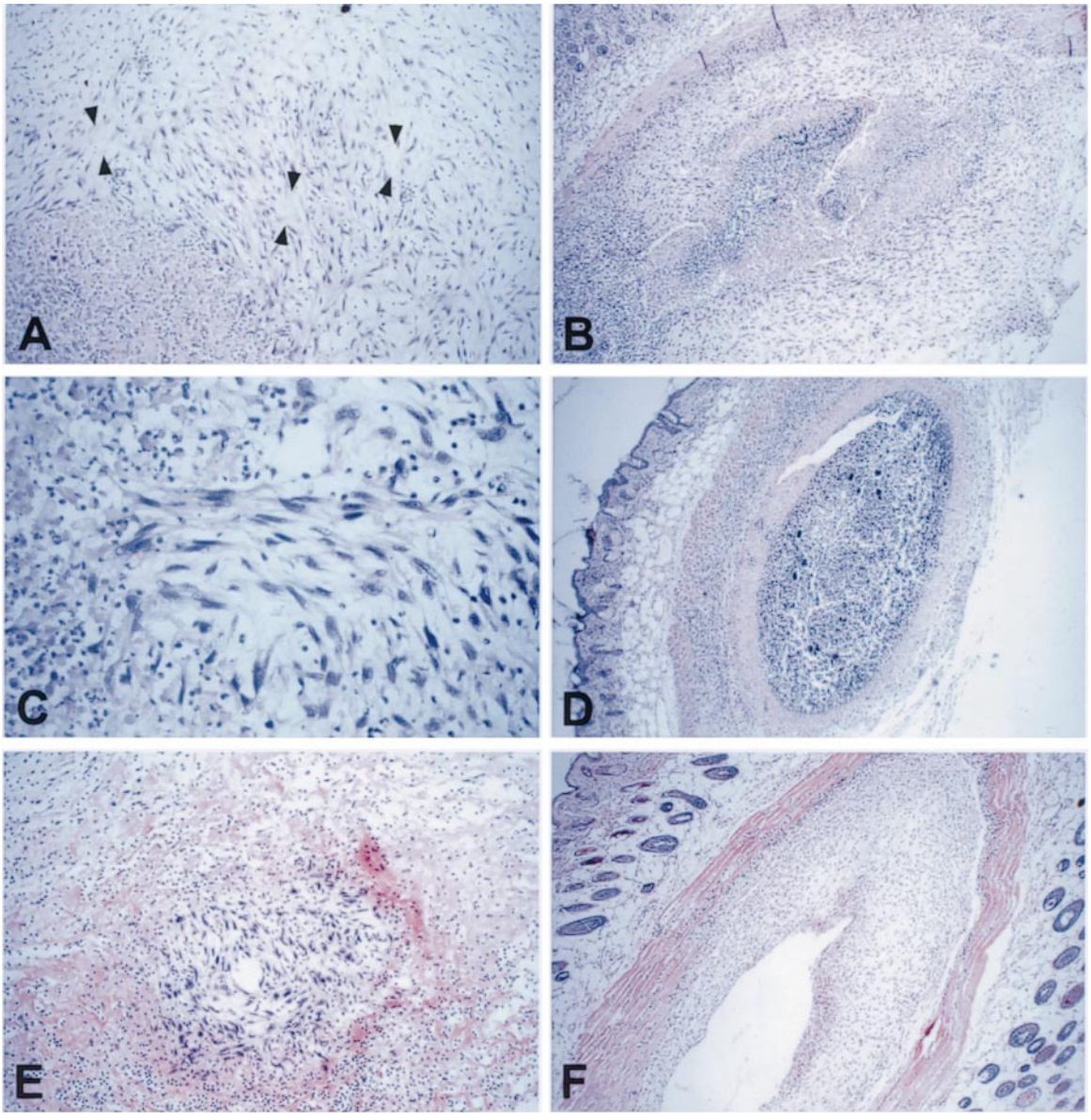


**Fig. 4.** Histological examination of tumors developed in nude mice inoculated with BBKI cells. Mice were killed during the period of tumor growth and regression. After a short phase of tumor growth with the presence of new capillaries in the peritumoral tissue (**A**, arrowheads), the tissue around the tumor is gradually infiltrated by fibroblasts and mononuclear cells (**B**), which start to invade the tumor mass (**C**). A thick fibrous wall then develops around the tumor and necrotic areas appear among tumor cells (**D**). The final stages of tumor regression are characterized by replacement of the tumor mass by fibroblasts and mononuclear cells (**E**) or by complete degeneration of the neoplastic tissue due to progression of tumor cell necrosis: in this case, an empty cavity remains in the place of the tumor, surrounded by the fibrous wall (**F**). H&E; original magnifications: ×125 (A and E); ×50 (B, D and F); ×300 (C).

the formation of a fibrous tissue. To this purpose, we investigated the activity of IL-6 and plasminogen activator (PA) as well as the expression of basic fibroblast growth factor (bFGF) in six BBKI clones. We first tested the activity of IL-6 on proliferation of human fibroblasts. Different results were obtained in the six clones analyzed: two clones did not express IL-6, three clones expressed values comparable with MHE control cells, whereas one clone (BBKIcl5) showed a high expression of IL-6 (data not shown). Nevertheless, BBKIcl5 behaved as the other transformed cell lines in the induction and regression of tumors. We then evaluated bFGF production of BBKI clones by western blot analysis, using specific anti-bFGF antibodies. The six clones expressed different quantities of bFGF, ranging between 21 and 68 ng/mg protein, whereas the MHE control cells expressed 67 ng/mg protein. However, BBKIcl5 expressed a significantly greater amount of bFGF (167 ng/mg protein) than that of the other clones. The same results were obtained in the assay for PA activity, since a correlation was observed between the values of PA activity and the expression of bFGF detected in cell clones. Since the transformed cell lines examined express different amounts of IL-6, bFGF and PA, these factors do not seem to be correlated to tumor regression.

We therefore studied the production of other fibrogenic factors by BKV-transformed cells. Human fibroblasts were seeded at the density of $1 \times 10^3$ cells/well in 96-well plates. These cells showed a 9- or 10-fold increase in cell number over a period of 8 days when stimulated with CM obtained from BBKIcl6 or Brain-BKcl1 cell cultures, respectively (Figure 5). The stimulatory activity was concentration dependent. The same enhancing effect was observed when cell motility was evaluated with a chemotaxis microchamber assay. The addition of CM to the lower wells of the chamber stimulated motility of human fibroblasts and NHI3T3 cells, again in a dose-dependent manner (Figure 6). These results suggest that BKV-transformed cells release factors that activate both proliferation and migration of human and mouse fibroblasts.

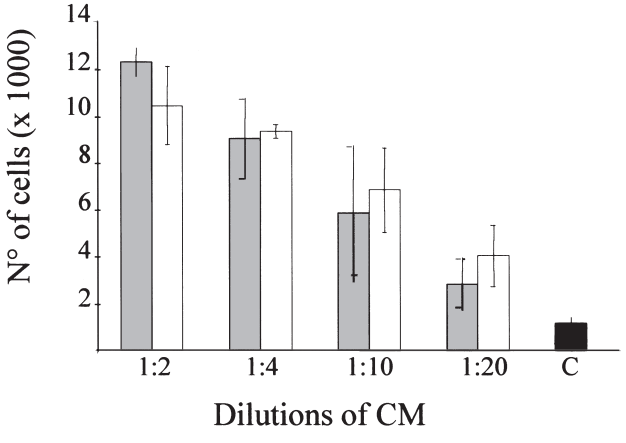


**Fig. 5.** Effect of CM from BBKIcl6 and Brain-BKcl1 cells on the proliferation of human fibroblasts. Human fibroblasts were seeded in 96-well plates ($1 \times 10^3$ cells per well) for 24 h in presence of DMEM containing 10% FCS, washed and exposed to CM of BBKIcl6 (filled columns) or Brain-BKcl1 (non-filled columns). CM was diluted with DMEM and cells were counted after 8 days. The control (C) is represented by human fibroblasts maintained in undiluted CM from normal endothelial cell cultures. The data shown are means ± SD of four determinations in a representative experiment.

*Role of TGF-β1 in activation of fibroblast proliferation and migration*

TGF-β1 is a fibrogenic (41) and angiogenic (42) cytokine, which activates the proliferation and migration of fibroblasts, lymphocytes and monocytes (43–47). Thus, we analyzed whether TGF-β1 is expressed in BKV-transformed cells. The analysis of the TGF-β1 transcript was carried out by hybridization of total cytoplasmic RNA from BBKI and BK-MHE cell clones to a $^{32}$P-labeled TGF-β1 cDNA probe. A band of 2.5 kb, representing the major mouse transcript of TGF-β1 (48) was detected both in BBKI and in BK-MHE cells (Figure 7, lanes 1–4 and 6 and 7). A TGF-β1-specific transcript

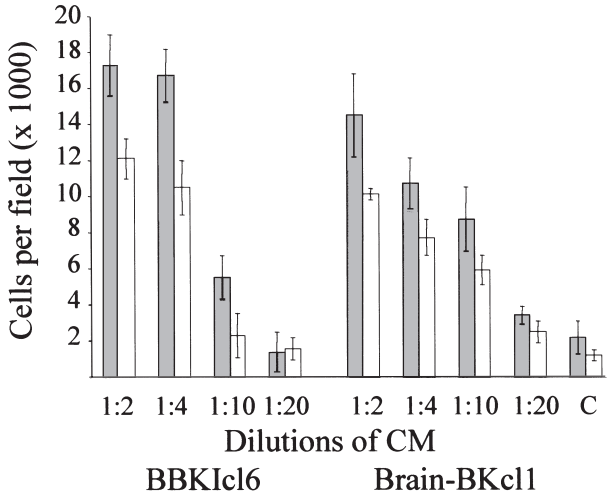


**Fig. 6.** Effect of CM of BBKIcl6 and Brain-BKcl1 on migration of fibroblasts (filled columns) and NIH3T3 cells (non-filled columns). Migration of cells was measured by a chemotaxis microchamber assay. CM from BKV-transformed cells, was placed in the lower wells of the chamber and $7.5 \times 10^4$ human fibroblasts or NIH3T3 cells were seeded in the upper wells. After 4 h of incubation, cells migrated to the lower surface were counted. The values are means ± SD of the number of cells counted in five fields in a typical experiment. As a control (C), undiluted CM from normal endothelial cell cultures was placed in the lower wells of the chamber.

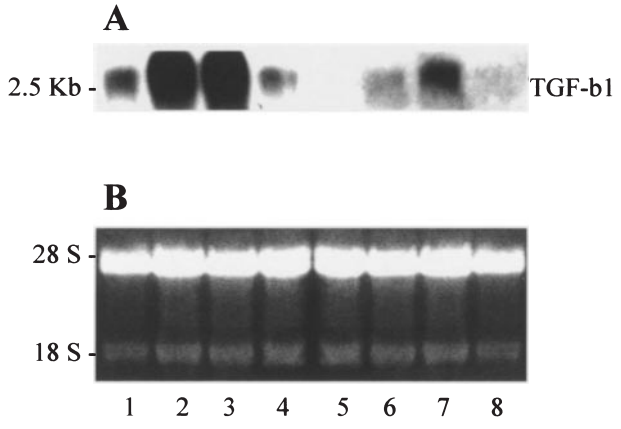


**Fig. 7.** Northern blot analysis of total cytoplasmic RNA extracted from endothelial cells. RNA (20 μg) was migrated in agarose gels and hybridized to a $^{32}$P-labeled TGF-β1 cDNA probe. (**A**) Lane 1, BBKIcl9; lane 2, BBKIcl7; lane 3, BBKIcl6; lane 4, BBKIcl5; lane 5, normal brain endothelial control cells; lane 6, BK-MHE cl3; lane 7, BK-MHE cl1; lane 8, MHE spontaneously immortalized control cells. (**B**) Ethidium bromide-stained nylon membrane to demonstrate even loading and transfer. This experiment is representative of three experiments performed with similar results. The position of 28S and 18S ribosomal RNAs is indicated on the left.

**Table III.** Effect of an antibody to TGF-β1 on proliferation and migration of human fibroblasts and NIH3T3 cells cultured in CM from BKV-transformed cells

| Sample[a] | Proliferation[b] no. of cells (×1000) | Migration[b] no. of cells per five fields | |
|---|---|---|---|
| | Human fibroblasts[c] | Human fibroblasts[c] | NIH3T3 cells[c] |
| Control | 1.8 ± 0.1 | 22 ± 9.0 | 12 ± 3.0 |
| CM from normal endothelial cells | 2.3 ± 0.6 | 43 ± 7.0 | 21 ± 8.0 |
| CM from endothelial cells + anti-TGF-β1 Ab | 2.0 ± 0.7 | 39 ± 6.0 | 15 ± 11 |
| CM from endothelial cells + rabbit IgG | 2.8 ± 0.9 | 35 ± 10 | 19 ± 4.0 |
| CM from BBKIcl6 | 9.8 ± 1.0* | 145 ± 23* | 107 ± 18* |
| CM from BBKIcl6 + anti-TGF-β1 Ab | 3.3 ± 1.1§ | 55 ± 16§ | 34 ± 8.0 |
| CM from BBKIcl6 + rabbit IgG | 10.5 ± 2.1 | 133 ± 11 | 114 ± 21 |
| CM from Brain-BKcl1 | 8.9 ± 0.8* | 121 ± 13* | 111 ± 20* |
| CM from Brain-BKcl1 + anti-TGF-β1 Ab | 5.2 ± 1.3§ | 54 ± 6.0§ | 87 ± 5.0§ |
| CM from Brain-BKcl1 + rabbit IgG | 8.0 ± 1.1 | 121 ± 8.0 | 139 ± 14 |
| Recombinant TGF-β1 | 7.3 ± 1.7* | 96 ± 13* | 67 ± 8.0* |
| Recombinant TGF-β1 + anti-TGF-β1 Ab | 1.7 ± 0.9§ | 21 ± 8.0§ | 17 ± 4.0§ |
| Recombinant TGF-β1 + rabbit IgG | 8.0 ± 0.4 | 96 ± 13 | 67 ± 8.0 |

[a]Ab, rabbit polyclonal anti-TGF-β1 antibody; IgG, rabbit immunoglobulin.
[b]Data were analyzed by one-way analysis of variance and Student–Newman–Keuls test. *$P < 0.05$ within CM from BKV-transformed cells or human recombinant TGF-β1 (5 ng/ml); §$P < 0.005$ within CM or recombinant TGF-β1 treated with an anti-TGF-β1 antibody or with irrelevant rabbit IgG.
[c]Means ± SD of four determinations done in a typical experiment out of three performed with CM prepared from different passages of the transformed cell clones. The CM from normal murine endothelial cells was prepared from cells at the second passage.

was not detected in normal murine endothelial cells (Figure 7, lane 5), and was expressed at low levels in MHE control cells (Figure 7, lane 8). Densitometric analysis showed that BBKIcl9, BBKIcl7, BBKIcl6 and BBKIcl5 expressed, respectively, 4.8-, 24.6-, 25.8- and 4.3-fold as much TGF-β1 transcript as normal brain endothelial cells. Similarly, BK-MHEcl3 and BK-MHEcl1 transformed clones expressed 2.3- and 4.2-fold more TGF-β1 transcript than MHE control cells.

To establish if TGF-β1, expressed by BKV-transformed cells, is released in culture supernatants and is responsible for cell activation, we evaluated the effect of an antibody to TGF-β1 on the proliferation and migration of human fibroblasts and murine NIH3T3 cells treated with CM of BKV-transformed cells. When human fibroblasts and NIH3T3 cells were cultured with CM obtained from BBKIcl6 and Brain-BKcl1 or with 5 ng/ml of human recombinant TGF-β1, we observed an increase in proliferation and migration of these cells (Table III). If CM or recombinant TGF-β1 were pre-incubated with a polyclonal rabbit antibody to TGF-β1, both effects were greatly reduced (Table III). These results indicate that TGF-β1 is released by BKV-transformed cells and activates human fibroblasts and NIH3T3 cells. A direct role of TGF-β1 in regression of tumors induced by BKV-transformed cells was confirmed by the remarkable expression of TGF-β1 in the phase of regression of tumors induced by BKV-transformed cells (Figure 8A and B), whereas progressing tumors induced by MHE control cells did not express TGF-β1 (Figure 8C).

## Discussion

These experiments were undertaken to test a possible modification in the behavior of endothelial cells after BKV transformation and to establish an experimental model of BKV-induced KS. This approach was justified by the following evidence: (i) the angiogenic activity of SV40 Tag (24,49) and the great homology (80%) between SV40 and BKV Tags (50); (ii) the presence of BKV early region sequences in KS tissue (23); (iii) the possibility that BKV may be sexually transmitted (23) and therefore fulfill the properties of the putative infectious agent that has been proposed to participate in the pathogenesis of KS (2).

BKV transforms mouse endothelial cells, but transformed cells do not express endothelial markers, suggesting that BKV transformation leads to loss of differentiation. Because of the high prevalence of endothelial cells in the primary cultures of brain cells, it is unlikely that BKV transformation had involved other cell types such as fibroblasts or glial cells. BKV-transformed endothelial cells were tumorigenic in nude mice, but tumors constantly regressed in a variable period of time. This result is unexpected and was never observed before in studies of BKV-transformation, because BKV-transformed hamster and mouse fibroblasts and glial cells induce, in syngeneic animals or in nude mice, tumors, which invariably progress to malignancy and bring to death the inoculated animals (51–54). Histological examination indicated that, after a phase of tumor growth, the peritumoral tissue, initially showing neoformed capillaries, evolved towards the formation of a fibrous wall devoid of vessels, but rich in fibroblasts and mononuclear cells. Mononuclear cell infiltration of tumors, lack of vascularization and nutritional starvation led to tumor cell necrosis and complete degeneration of the neoplastic tissue. This morphological pattern is suggestive of production of pro-fibrotic cytokines by BKV-transformed cells. Indeed, BKV-transformed endothelial cells were found to express a great amount of TGF-β1 transcript and their CM promoted proliferation and migration of human fibroblasts and NIH3T3 cells, which was inhibited by a specific antibody to TGF-β1. TGF-β1 was produced during the regression of tumors induced by BKV-transformed cells, while its expression was absent in progressing tumors induced by MHE control cells. TGF-β1 is therefore probably responsible for the production of a fibrotic tissue around tumors induced by BKV-transformed cells. This conclusion is supported by observations concerning other human and experimental tumors where fibrosis, induced by TGF-β1, develops during tumor evolution (55–57).

The mechanism of TGF-β1 induction by BKV is at present unclear. Since this effect is produced by transformation of endothelial cells with BKV early region, it is reasonable to

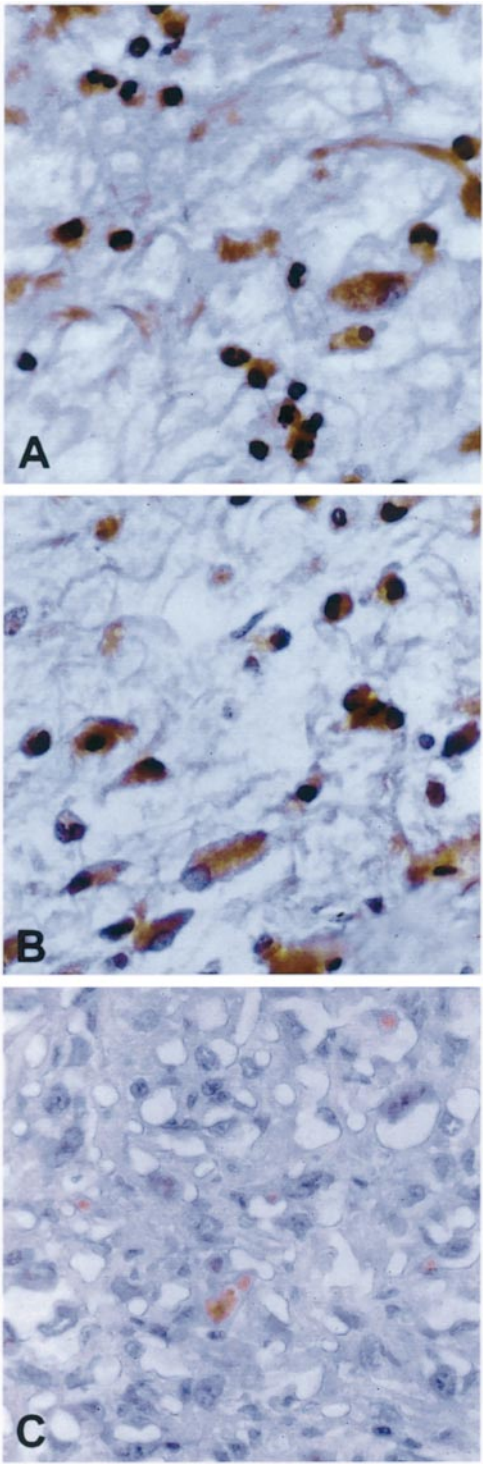


**Fig. 8.** TGF-β1-expression in tumors induced by BBKIcl1, BBKIcl9 and control MHE cells in nude mice. ABC immunohistochemistry was performed as described in Materials and methods. (**A** and **B**) Tumors induced by BBKIcl1 and BBKIcl9 cells; (**C**) tumor induced by control MHE cells. Tumors induced by BBKIcl1 and BBKIcl9 were removed during the phase of regression, whereas the tumor induced by MHE control cells was growing and progressing. Original magnification ×500.

propose that expression of TGF-β1 is induced by BKV TAg. Transformation of endothelial cells by BKV TAg mutants could clarify this hypothesis. Since tumors induced by BKV-transformed mouse fibroblasts do not regress, the peculiar role of the endothelial cells in participating in TGF-β1 induction should be investigated by analyzing the regulation of TGF-β1 gene expression in fibroblasts and endothelial cells.

Concerning the role of TGF-β1 in the initial phase of our model, it is interesting to note its possible bi-functional activity in angiogenesis. It has been reported that TGF-β1 is angiogenic *in vivo* (42,45), and homozygous knock out mice for TGF-β1 (58) or endoglin (59) have defective vascular development. Endoglin is a TGF-β binding protein expressed on the surface of endothelial cells (59). In contrast, TGF-β1 is anti-angiogenic in an experimental model of gallbladder tumor (60). *In vitro*, TGF-β1 inhibits both the proliferation and migration of endothelial cells in monolayer culture, but it promotes organization of cells into tube-like structures in a three-dimensional gel of matrix proteins (61–64). Finally, TGF-β1 activates the expression of angiogenic inducers such as bFGF and vascular endothelial growth factor (65,66), but down-regulates the expression of vascular endothelial growth factor receptor-2 (67). In view of these different effects, TGF-β1, secreted by BKV-transformed cells, may have initially induced formation of vessels around the tumors, then shifting the whole process towards inhibition of angiogenesis and fibrosis. TGF-β1 is also a potent chemotactic agent, attracting lymphocytes, monocytes and macrophages (45–47).

Therefore, the complete degeneration of the tumor mass in our model may be due to four factors, all dependent on host functions and attributable to the effects of TGF-β1: (i) infiltration of tumors by inflammatory cells, causing destruction of the neoplastic tissue; (ii) inhibition of angiogenesis; (iii) proliferation of fibroblasts and consequently; (iv) production of a fibrous wall around tumors which hindered the supply of oxygen and nutrients to tumor cells. It seems reasonable to exclude an immunological rejection of tumors, because BKV-transformed murine endothelial cells were implanted in immunologically deficient nude mice where BKV-transformed mouse fibroblasts induced progressively growing malignant tumors (54). The human counterpart of this model may be represented by those KS cases where the tumor spontaneously regresses (68) and by some vascular tumors where an over-expression of TGF-β1 was detected (69,70).

Regression of tumors induced by BKV-transformed murine endothelial cells was therefore an unexpected result and could represent a model of tumor dormancy (71). In fact, tumor encapsulation and fibrosis are signs of a dormant state in some experimental tumors (72,73) and induction of dormancy was proposed as a therapeutic approach to arrest tumor progression (71). In addition, the experimental angiogenic model of KS based on BKV is not fulfilled by our data. Infection of human KS tissue by BKV, however, may be responsible for the portion of KS cases where the tumor spontaneously regresses. Indeed, the histologic pattern of KS regression in humans is similar to what we observed in mice, since disappearance of the neoplastic tissue is characterized by mononuclear cell infiltration and proliferation of fibroblasts, accompanied by formation of a fibrotic tissue (74,75). It would be interesting to assess if the process of KS regression in humans is associated with production of TGF-β1 in the neoplastic tissue. If this is the case, KS regression could be induced or accelerated by any means, which enhances TGF-β1 production at the tumor site. Finally, expression of BKV early region in endothelial cells of other malignant human tumors may hinder tumor progression or induce regression by over-expression of TGF-β1.

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