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SIMIAN SARCOMA VIRUS TRANSFORMATION OF NORMAL RAT KIDNEY FIBROBLASTS IS ASSOCIATED WITH MARKEDLY INCREASED BASIC FIBROBLAST GROWTH FACTOR EXPRESSION

Peter G. Milner

Department of Medicine,
Jewish Hospital at Washington University Medical Center
St. Louis, MO 63110

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Transformation of normal rat kidney fibroblasts (NRK) by the simian sarcoma virus (SSV) occurs as a result of expression of $p28^{v\text{-}sis}$, a homologue of platelet-derived growth factor-B chain. Chromatographic separation revealed that the bulk (85%) of the mitogenic activity in SSV-transformed NRK cells was not due to $p28^{v\text{-}sis}$ but rather two distinct endothelial cell growth factors that eluted off heparin-Sepharose between 1 and 2 M NaCl. Protein purification and Northern blot analysis revealed that one of these growth factors was the 18 kd form of bFGF, the expression of which was found to increase 15-fold with SSV-transformation of NRK cells. The pure 18 Kd bFGF had no effect on NRK cell growth but was a potent neurotrophic agent for fetal rat cortical neurones and a potent growth factor for fetal bovine heart endothelial cells, suggesting a paracrine but not autocrine role for this protein. The second endothelial cell growth factor activity in SSV-transformed NRK cells was due to an 18 Kd protein which could be distinguished immunologically, biochemically, and mitogenically from bFGF.

Sarcomas are highly vascular tumors with extensive formation of new, poorly formed blood vessels. This vascularity leads to two recognized complications of sarcomas, hemorrhage and hematogenous spread. To investigate what angiogenic growth factors are produced by sarcomas and may induce these changes, we chose as a model the simian sarcoma virus (SSV)-transformed normal rat kidney (NRK) fibroblast.

The v-sis oncogene of the acutely transforming SSV is responsible for its tumorigenic properties (1). Protein sequencing of human platelet-derived growth factor (PDGF) reveals 92% homology between the PDGF B-chain and $p28^{v\text{-}sis}$, the gene product of the v-sis oncogene (2). $p28^{v\text{-}sis}$ is mitogenic for mesodermal cells but not angiogenic (3).

SSV-transformed NRK cells are known to express growth factor activities other than $p28^{v\text{-}sis}$, including those which elute in the 1-2 M NaCl range off heparin-Sepharose (4). In view of these data, and to investigate the role of angiogenic growth factors in sarcomas, we confirmed by protein purification using an endothelial cell mitogen assay, and by N-terminal amino acid sequencing, that the 18 kD form of bFGF is one of the major growth factor activities expressed

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by SSV-NRK cells. Another unique endothelial cell growth factor activity that bound more tightly to cation exchange resins than bFGF was detected at high levels in SSV-NRK cells and was shown to be due to an 18 kD protein distinct from bFGF.

MATERIALS AND METHODS

Cell Culture, Lysis and Subcellular Fractionations. NRK and SSV-NRK fibroblasts, obtained from ATCC and Dr. S. Aaronson (5) respectively, were grown to confluence in Dulbeco's Modified Eagles Medium (DMEM) plus 10% fetal calf serum (FCS), followed by serum free DMEM/F12 for 72 hours prior to harvesting in PBS plus 2 mM EDTA and lysis by nitrogen cavitation (1250 PSI) at 4°C. Lysates were centrifuged at 650 g for 45 minutes to pellet the nuclei; the supernatant was further fractionated by centrifugation at 100,000g for 1 hour to pellet the membrane fraction (P 100) and the supernatant cytosolic fraction (S100). The S100 fraction represented 93% of the mitogenic activity in SSV-NRK whole cell lysates while the remaining 7% was recovered in the P100 fraction. The S100 fraction was used throughout this study. Protein was assayed according to published methods (6).

Mitogen Assays. The mitogenic activity of samples was assayed in triplicate by measuring the incorporation of [methyl-^{3}H] thymidine into acid-insoluble DNA of confluent serum-starved NRK cells, and fetal bovine heart endothelial (FBHE) cells in 10% FCS. Unstimulated cells were used as background and background values were subtracted from all values shown.

Northern Blot Analysis. Messenger RNA was extracted from confluent plates of serum-stimulated SSV-NRK and NRK cells, using the guanidine isothiocyanate protocol, electrophoresed on 1% agarose formaldehyde gels, and transferred to nitrocellulose according to the methods of Maniatis et al. (7). The bovine acidic and basic FGF cDNA probes were a gift from Dr. Judith Abraham (California Biotechnology, Inc.). Probes were labeled using ^{32}p-dCTP (NEN), a random primer DNA labelling system (BRL, Inc.), to a specific activity of 6.6×10^8 cpm/μg of DNA. Nitrocellulose filters were pre-hybridized and hybridized to the probes (1.5×10^6 cpm/ml) as previously described (7). Filters were exposed to x-ray film (Xomat AR5) with intensifying screens at -70°C for up to 3 days before development.

Separation of Growth Factors Activities from SSV-NRK Lysates. Separation of the mitogenic activities in both cell line lysates were obtained by applying 160 mg of cell lysates in 500 ml buffer A onto a 2.5 x 45 cm SR 25/45 FPLC column packed with heparin-Sepharose (bed volume 30 ml), extensive washing with 20 mM Hepes pH 7.0, and elution with stepwise application of 3 column volumes (90 mls) of 0.5 M, 1 M and 2 M NaCl in 20 mM Hepes pH 7.0. Each fraction was collected and assayed for mitogenic activity as described above. The p28$^{v\text{-}sis}$ used to identify the precise molarity of NaCl at which this protein elutes off heparin-Sepharose was a gift of Dr. Arlen Thomason (Amgen).

Endothelial Cell Growth Factor Purification. The angiogenic growth factor activities eluting in the 0.9 to 2 M NaCl range off heparin-Sepharose were purified by modification of a previously described method (8) using the FBHE cell mitogen assay. 2L of the lysates containing 4g protein was applied to a 5 x 12.5 cm heparin-Sepharose column equilabrated at 0.9M Nacl in 20 mM Hepes pH 7.0, washed, and then eluted in 600 ml of 2 M NaCl in 20 mM Hepes pH 7.0. The eluate was dialyzed in 20 mM MES pH 6.0 overnight, loaded onto an HR 10/10 Mono S column and eluted in 20 ml 2M NaCl, in 20 mM MES pH 6.0. The 20 ml eluate was dialyzed overnight into 20 mM MES pH 6.0 before reloading onto a HR 5/5 mono S column and eluted by applying a linear 0-2 M NaCl gradient in 40 ml 20 mM MES pH 6.0. Fractions were assayed in both the FBHE and NRK cell mitogen assays.

Neurite Outgrowth Assay. The 18 kD bFGF purified from SSV-NRK cells was assayed for neurotrophic activity according to a previously described method (9).

RESULTS

SSV Transformation Leads to Increased Non-PDGF Growth Factor Expression.

The lysates of SSV-NRK fibroblasts contained 5.3 times more mitogenic activity than NRK lysates (Table 1). As expected there was a doubling of mitogenic activity from SSV-NRK cells eluting in 0.5-1.0M NaCl, due most likely to increased expression of $p28^{v\text{-}sis}$, since $p28^{v\text{-}sis}$ was shown to elute off heparin-Sepharose at 0.6M NaCl, and because all of the PDGF-immunoreactive proteins were shown to elute in the 0.5-1.0M NaCl fractions (data not shown).

A 12-fold increase in mitogenic activity eluting in the 1.0-2.0M NaCl fraction of SSV-NRK lysates was observed when compared to NRK lysates. Angiogenic growth factors are known to elute off heparin-Sepharose in the 1.0-2.0M NaCl range (10,11); therefore, we sought to determine by protein purification using an endothelial cell mitogen assay (FBHE cells) which angiogenic growth factors are expressed at high levels in SSV-NRK cells.

Purification of Endothelial Cell Growth Factors from SSV-NRK Cell Lysates.

The elution profile of protein, mitogenic (NRK), and angiogenic (FBHE) activity from the final column, a Mono S HR5/5 column, are shown in Figure 1. One major peak of angiogenic activity was due to a homogeneous 18 kD protein which eluted off Mono S between 0.5 and 0.8 M NaCl at pH 6.0. The silver stained appearance of this protein following SDS-Polyacrylamide gel electrophoresis is shown in Figure 2, as is its N-terminal amino acid sequence confirming that this activity was due to the 18 kD form of basic FGF. An 11,000-fold purification with a total recovery of 5% of the initial angiogenic activity was obtained in the purification of bFGF from SSV-NRK cells.

A second peak of angiogenic activity was also present. This activity bound more tightly to mono S, eluting between 0.8 and 1.1 M NaCl, and as shown in Figure 1, was distinct from bFGF because it was active in both the NRK and FBHE cell mitogen assays. Further purification using reversed-phase C_4 microbore chromatography revealed that this activity was due to an 18 kD protein

TABLE I. Heparin-Sepharose Separation of Mitogenic Activity in NRK and SSV-NRK Fibroblasts

	Cell Lysate*	0-0.5M	0.5-1.0M	1.0-2.0M NaCl	Recovery
NRK	63+ (100%)	16	12	18	46 (73%)
SSV-NRK	288 (100%)	13	25	220	258 (89%)
Fold Increase with SSV Transformation	5.3	1	2	12	

*160 mg protein both cell lines

+Total units of mitogenic activity in disintegrations per minute x 10^6 in NRK mitogen assay. These data were obtained by calculation of the 50% maximal point on a dose response curve, and represent the mean of five separate batches.

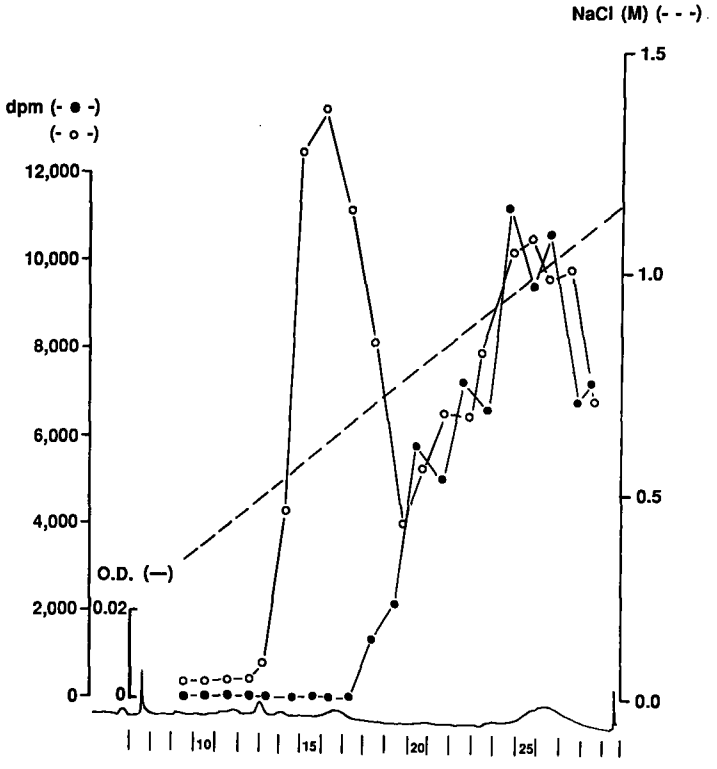
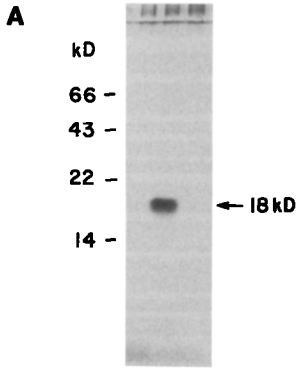


Figure 1. Mono S chromatographic separation of the mitogenic activity in SSV-NRK Cell lysates which binds to heparin-Sepharose between 0.9 and 2M NaCl. Each 1 ml fraction off the HR 5/5 Mono S column was assayed in both the NRK (closed circles), and the FBHE (open circles) cell mitogen assays. Shown are the activities in these 2 assays, the O.D. of the eluted proteins, and the NaCl gradient.



B

bFGF: PRO-ALA-LEU-PRO-GLU-ASP-GLY-GLY

18kD: X - X -LEU-PRO-GLU-ASP-GLY-GLY

Figure 2. bFGF purified from the SSV-NRK cell lysate. Shown in panel A is the SDS-Page and silver stained appearance of 40 μl of fraction number 16 off the Mono S HR 5/5 shown in Figure 1. Panel B shows the N-terminal amino acid sequence obtained from this tube, and the known sequence of rat bFGF for comparison.

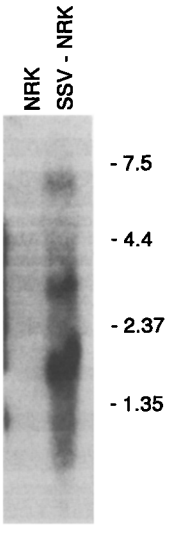


Figure 3. Increased bFGF gene expression in NRK cells transformed by the SSV. Shown is the Northern blot analysis of 20 μg of total mRNA from NRK cells and SSV-NRK cells respectively. Blots were probed with a radiolabeled bovine bFGF and autoradiograms developed after 3 days.

immunologically distinct from bFGF that required 51% acetonitrile (ACN) in dH_2O, 0.1% Trifluroacetic acid (TFA) to elute off C_4; bFGF elutes at 43%.

Basic FGF Expression is Markedly Increased in SSV-NRK Compared to NRK Cells.

As seen in Figure 3, expression of the 6 Kb rat bFGF mRNA transcript (12) was barely detectable in 20 μg of total RNA extracted from NRK cells. In contrast, 20 μg of SSV-NRK cell mRNA contained approximately 15-20 times more of the 6 Kb bFGF mRNA, as well as prominent mRNA bands at 1.8 and 2.8 Kb. Western blot analysis confirmed a 15-fold increase in the level of 18 kD bFGF protein expression in SSV-NRK compared to NRK cells.

Increased bFGF expression in SSV-NRK cells did not appear to be due to PDGF-inducibility of bFGF since there was no increase in bFGF mRNA transcripts in RNA extracted from serum-starved NRK cells 1/2, 1, 3, 6, 18 and 24 hours after exposure to 100ng/ml PDGF (data not shown). In addition, comparable increases in bFGF were also observed in NRK cells transformed by a vector containing the v-sis oncogene alone, rather than the wild type SSV.

18kD Form of bFGF in SSV-NRK Cells Appears to Act as a Paracrine Factor.

The 18 kD form of bFGF purified from SSV-NRK cells was not mitogenic in the NRK mitogen assay, despite being a potent mitogen in the FBHE cell assay. Similar results were obtained with recombinant human and purified bovine brain bFGF. These data suggest that the 18 kD form of bFGF purified from SSV-NRK cells may act as a paracrine, but not an autocrine growth factor.

To further confirm that the 18 kD form of bFGF was biologically active following purification, the effect of this protein on dendrite outgrowth from 18 day fetal cortical rat neurons was studied. As shown in Figure 4, 200 ng

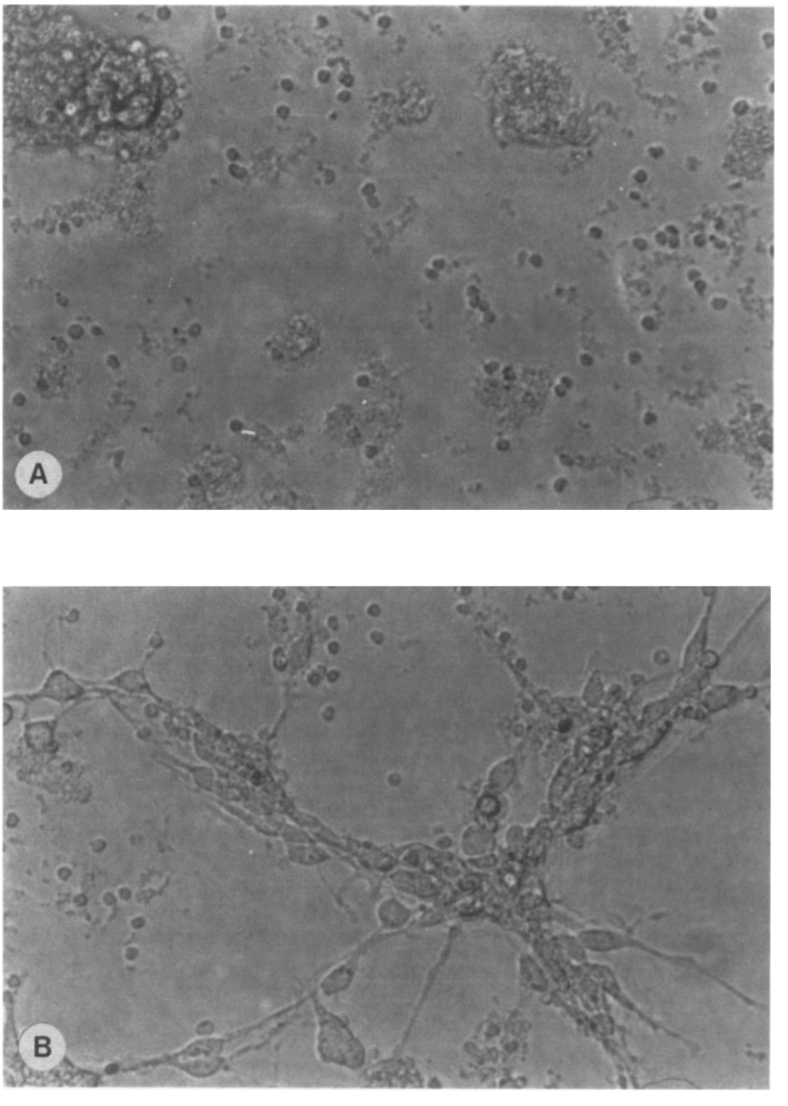


Figure 4. Neurite outgrowth induced by the 18kD form of bFGF purified from SSV-NRK cell lysates. Phase contrast photomicrographic views at 40 x magnification of 18 day fetal rat cortical neurons prepared as described elsewhere (9) and cultured for 48 hours in DMEM plus 0.1% BSA in wells on a 96 well Falcon tissue culture plate pretreated with either (A) 200 µl DMEM and 0.1% BSA for 1 hour, or (B) 30 µl PBS containing 200ng of the purified 18kD bFGF shown in Figure 2 for 2 hours, followed by 200 µl DMEM and 0.1% BSA for 1 hour.

per well of the pure 18 kD form of bFGF stimulated marked neurite outgrowth at 48 hours as previously described (13), confirming its neurotrophic activity in the same species. These data could account for the observed neural invasion also seen in sarcomas, an activity first noticed in sarcoma extracts (14) and presumed, but never proven, to be due to nerve growth factor (NGF) expression.

DISCUSSION

Cellular transformation is a complex process which in the acutely transforming SSV can be attributed directly to one key event, the expression of

the v-sis oncogene (15). Although v-sis expression is fundamental, it is likely that the transformed phenotype is determined by many other cellular events. Transformation is associated with changes in cell morphology and metabolism. We have been able to identify considerable changes in two heparin-binding growth factor activities which occur in association with SSV transformation of NRK fibroblasts, and that the majority (85%) of mitogenic activity present in SSV-NRK cell lysates was due to these two activities rather than to $p28^{v\text{-}sis}$. Both growth factor activities proved to be mitogenic for endothelial cells. One of these activities has been purified to homogeneity and was shown by N-terminal amino acid sequence, Northern and Western blot analysis to be due to increased expression of the 18 kD form of the bFGF protein.

Conditioned media obtained from cultured SSV-NRK cells reportedly contain an activity which induces down-regulation of the epidermal growth factor receptor and was provisionally characterized as a member of the fibroblast growth factor family because it induced autophosphorylation of the bFGF receptor (16). It was also recognized that bFGF, if expressed at sufficiently high levels, can induce transformation of fibroblasts even in the absence of a signal peptide (17). From these data it is clear that over-expression of bFGF by SSV-NRK cells described in this study could contribute to the transformed phenotype by an autocrine or paracrine mechanism independent of the expression and secretion of $p28^{v\text{-}sis}$.

Increased bFGF expression has previously been observed in several cell lines (18,19), though its significance has not been appreciated. We have shown that the 18 kD bFGF protein expressed at high levels in SSV-NRK sarcoma cells, stimulates growth in a paracrine fashion of endothelial cells and neurones which could lead to the neural and vascular invasion typically seen in sarcomas. We also have shown that this protein appears to have no effect on the growth of NRK cells, the parent cells of the tumor. These data suggest that the 18 kD bFGF protein induced by sarcomatous change may have a paracrine, but not an autocrine role in tumors due to SSV-NRK cells. A second mitogenic activity was detected in SSV-NRK cells which bound more tightly to mono S and appeared to be due to a 18 kD protein immunologically distinct from bFGF. This protein stimulated both endothelial cell division and proliferation of NRK cells and so may act as both an autocrine and paracrine growth factor.

The increase in the level of expression of the bFGF gene in SSV-NRK and v-sis transformed cells compared to levels of expression of bFGF in either serum-starved or PDGF-stimulated NRK cells, is similar to the observation of Iberg et al. (19) that transformation of rat-1 fibroblasts by the H-ras oncogene is associated with a 2 to 4 fold increase in expression of both the 18 and 22 kD form of bFGF protein. In SSV-NRK cells, however, unlike H-ras transformed Rat-1 fibroblasts, only the 18kD form of bFGF is over-expressed following oncogenic transformation since Western blot analysis and protein purification failed to detect the 22 kD form of bFGF.

We have determined that the increased expression of angiogenic growth factors observed in association with transformation is dependent upon expression of the v-sis oncogene rather than simply a consequence of retroviral expression, as is known to be the case with at least one other growth factor, Transforming Growth Factor-alpha (20). PDGF inducibility of the bFGF gene was not demonstrated, suggesting that increased bFGF expression in SSV and v-sis transformed NRK cells occurs by a novel as yet undefined mechanism, and not due simply to $p28^{v\text{-}sis}$-PDGF receptor interaction.

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