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Characterization of Tumors Produced by Signal Peptide-Basic Fibroblast Growth Factor-Transformed Cells

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Basic fibroblast growth factor (bFGF) is found in a variety of cells and tissues. We have previously shown that bFGF is a transforming growth factor, but only when fused to a signal peptide (sp-bFGF). Cells expressing the native bFGF are tumorigenic in nude mice only, where the tumors form at a low frequency and grow very slowly as compared to sp-bFGF tumors. The cells transformed by the sp-bFGF growth factor gene cause rapidly growing tumors within 10 days in 100% of syngeneic and nude mice. In nude mice, the tumors are highly vascularized, while the vascularization in immunocompetent syngeneic mice is not as prominent. The syngeneic mice have a characteristic humoral immune response to sp-bFGF tumors, which differs from that mounted against *ras*-induced tumors. The ability of bFGF to induce tumorigenicity is significant in view of the recent discoveries of three new oncogenes: *hst*, *int-2*, and an oncogene from a human colon cancer. In addition to homology with FGF, the proteins encoded by these oncogenes all have a potential signal peptide at the protein's amino terminus, suggesting a mode of action analogous to that of our artificial signal peptide-bFGF (sp-bFGF) transforming growth factor model system.

Key words: autocrine transformation, angiogenesis, bFGF, heparin, oncogene, tumorigenicity, signal peptide

A number of distinct growth factors have been found associated with tumor cells. These growth factors have been associated with several properties of the tumor mass including angiogenesis—the generation of new capillary beds. Although the natural triggers of this process are unknown, neovascularization can be induced in certain in vivo models by several mitogens, including the basic fibroblast growth factor (bFGF) [1]. bFGF, originally isolated from brain and pituitary, is found in richly vascularized tissues such as placenta, retina, kidney, corpus luteum, and numerous solid tissues, as well as in vascular endothelial cells in culture. Analysis of bFGF synthesis in cell culture indicates that bFGF is primarily a cell-associated

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growth factor. This lack of secretion is consistent with the apparent lack of a consensus signal peptide domain in the bovine and human bFGF genes. It is conceivable that this absence of a well-defined signal peptide is responsible for its failure to be secreted.

A family of new oncogenes encoding bFGF-like proteins was recently isolated from a Kaposi sarcoma cell line (*hst*), several human stomach cancers (*hst*), a human bladder cancer, and mouse mammary gland tumors caused by MMTV virus (*int-2*)[2–6]. The proteins encoded by these oncogenes share about 45% amino acid homology with the basic and acidic FGF. In addition, they all code for potential amino terminus signal peptide sequences.

bFGF is mitogenic for a broad spectrum of cells; probably all cells of mesodermal and neuroectodermal origin express FGF receptors on their surfaces [1,7]. The autocrine model of transformation would predict that bFGF can act as a transforming agent in any of these cell lines as long as it has access to the receptor, activation of which would then lead to constitutive proliferation. Presumably, access to the cell surface receptor can be achieved via secretion of the growth factor, or by co-compartmentalization of the growth factor and its receptor [8–11].

We have recently generated a series of NIH-3T3-derived cell lines that stably express various levels of bFGF with and without an artificial signal peptide. Only the cell lines expressing the chimeric signal peptide-bFGF protein were morphologically transformed and tumorigenic in syngeneic mice [12].

The tumors formed by our chimeric sp-bFGF expressing cell lines may be potential models for those tumors from which the above-mentioned FGF-homologous oncogenes were isolated. In this report we characterize the model tumors in terms of bFGF production, vascularization, growth in syngeneic and nude mice, and the humoral immune response of the animal to the tumor.

MATERIALS AND METHODS

Cell Lines Expressing the Native and the Chimeric bFGF [12]

Cell lines expressing various amounts of the native or the chimeric bovine brain bFGF were generated as described previously [12]. In brief, mammalian expression vectors directing expression of the normal bFGF protein or of a chimera consisting of bFGF fused to the immunoglobulin heavy chain signal peptide were transfected into NIH-3T3 cells, and stable clonal cell lines were isolated. Control cell lines (NIH-CONT) transfected only with the dominant selectable marker pSV2-neo were simultaneously generated. The bFGF protein production by these cell lines was measured by the ability of cell lysates or conditioned media to stimulate DNA synthesis in Balb-c/3T3 cell cultures [13,14]. In parallel, a *ras*-transformed NIH-3T3 cell line, EJ 62 Bam 6a [15], was transfected with the above expression plasmids, and stable, neomycin-resistant native or chimeric bFGF-expressing cell lines were selected.

Cell line nomenclature is summarized as follows: NIH, derived by transfection of NIH-3T3 mouse fibroblasts; B, expresses the native bovine brain bFGF; IgB, expresses the chimeric sp-bFGF protein where the immunoglobulin signal peptide has been fused to the native bFGF; N, expresses the dominant selectable marker for neomycin resistance; #, number assigned to that particular clone; CONT, control cell line expressing neomycin resistance; EJ, transformed with the Ha-*ras* derived from the human EJ/T24bladder carcinoma cell line.

Heparin-Sepharose Affinity Chromatography [13,14]

The cells were grown until they reached confluence, and the conditioned medium was removed. The cells (about 10^8 per experiment) were resuspended at 10^7 per ml in 1 M NaCl, 10 mM Tris-HCl, pH 7.0, and lysed by sonication. The cell debris was removed by centrifugation, and the supernate was diluted to 0.1 M NaCl. This cell lysate was applied to 3 ml heparin-Sepharose columns, and bFGF was eluted with a 0.1 to 3 M NaCl gradient (80 ml). The collected fractions (4 ml) were assayed for stimulation of DNA synthesis in BALB-c/3T3 cells [13,14].

Western Blotting

Protein samples were resolved by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was probed with anti-bFGF antiserum, and the bound antibodies were visualized with ^{125}I-protein A. The anti-bFGF antisera were prepared by immunizing rabbits with synthetic peptides corresponding to positions 1–12 (amino-terminal) and positions 33–43 (internal) of bFGF [13].

Tumorigenicity Studies

Four- to 6-week-old, male NIH-3T3 syngeneic NIH/NSF mice were obtained from NCI, Frederick, MD. They were injected subcutaneously with 2×10^6 cells within a week of arrival. The athymic nude mice (NUDE/NUDE) were also obtained from NCI, Frederick, MD. The day prior to injection with 2×10^6 cells, the nude mice were irradiated with 145 rad/min for 4 minutes from a cobalt source. The animals were observed for tumor formation for 3–4 months.

Histology

The tumors were harvested when they reached the size of about 1 cm^3 and were fixed in 2.5% glutaraldehyde with 2% paraformaldehyde in 0.14 M cacodylate buffer, pH 7.4, for 2 hours at room temperature. The tumors were dehydrated in a graded series of alcohols and embedded in JB4 Plastic (Polysciences). Sections (2 μm thick) were cut on a Reichert 2050 Supercut microtome and stained with hematoxylin and eosin. The histological sections were photographed on a Zeiss photomicroscope.

Analysis of the Humoral Immune Response to bFGF Tumors in Syngeneic Mice

Mice were bled from their tails 2–4 weeks after injection with tumorigenic cells. The blood was allowed to coagulate on ice for 30 minutes, and the sample was centrifuged for 10 minutes at 4°C in an Eppendorf centrifuge. The serum was removed into a fresh container and stored at 4°C, where it has been stable for the last 10 months.

Immunoprecipitation (IP) was used to analyze the serum for specific antibodies to proteins in the producing tumor, as well as in the parental NIH-3T3 cells. IP was carried out as follows: NIH-IgBNM6–1 or NIH-3T3 cells were grown in Dulbecco's minimum essential medium (DMEM) (Gibco) supplemented with 10% calf serum, penicillin and strep tomycin to about 3×10^6 cells per 100-mm dish. The cells were radiolabeled for 12 hours with 5 ml cysteine-free DMEM (Gibco) supplemented with 5% dialyzed calf serum, penicillin, streptomycin, and 50–100 μCi of ^{35}S-cysteine (specific activity 1,000 Ci/mmol, New England Nuclear) per 100-mm dish. After

removal of media, the cells were washed twice with phosphate buffered saline (PBS), resuspended into 1 ml of cell lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, and 0.5% (v/v) Nonidet P40 detergent), and incubated on ice for 5 minutes. The nuclei were removed with a 5-minute centrifugation in an Eppendorf microfuge. The supernatant was incubated for 4–24 hours with 4 μl of either normal mouse sera or sera collected from a tumor-bearing mouse. Twenty microliters of protein A-Sepharose was added and incubated for 30–60 minutes. The pellet was washed four times with cell lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 1% aprotinin solution (Sigma). Twenty microliters of SDS-PAGE sample buffer was added to the washed pellet, and the sample was resolved by electrophoresis on SDS-PAGE [16].

RESULTS

Cell Lines Expressing Either the Native or the Chimeric bFGF Protein

NIH-3T3 fibroblasts express very low levels of endogenous bFGF, about 0.04 units/10^4cells [12]. (One unit is the concentration of bFGF required to stimulate half-maximal DNA synthesis in Balb-c/3T3 cells [13].) However, they are known to possess bFGF receptors on their cell surfaces and to respond mitotically to exogenous bFGF [13,14]. As described previously, expression plasmids directing production of either the native or the chimeric (signal peptide-containing [sp-]) bFGF were transfected into these cells [12]. This enabled us to generate a series of NIH-3T3-derived cell lines that express various levels of either the native bovine brain bFGF (0.4–4.6 units/10^4 cells), or the chimeric sp-bFGF in which the mouse heavy chain immunoglobulin signal peptide is fused to the amino terminus of the native bovine brain bFGF molecule. More than 20 independent monoclonal cell lines were analyzed for expression of the chimeric sp-bFGF. The highest level of growth factor activity was found to be about 0.8 units/10^4 cells. The chimeric sp-bFGF appears to be toxic to NIH-3T3 cells when produced in higher amounts [12].

Control cell lines and cell lines expressing equivalent levels of the native and the chimeric protein were chosen for further characterization by heparin-Sepharose chromatography and Western blot analysis. Figure 1A shows heparin-Sepharose affinity elution profile for the cell lysates of three selected cell lines. bFGF elutes from heparin-affinity column at a characteristic 1.5 M NaCl [13]. As expected, very little growth factor activity elutes from a column of NIH-CONT control cell lysate. In contrast, lysates of cell lines expressing either the native or the chimeric sp-bFGF both contain heparin-binding, biologically active growth factor that elutes from the column with 1.5 M NaCl. The fractions eluting at 1.5 M NaCl were probed in an immunoblot [14] using an antibody raised against an internal region of bFGF [13]. Purified recombinant bovine brain bFGF was analyzed in parallel for positive control. The results are shown in Figure 1B. Two transfected cell lines, NIH-BNM7 (transfected with the vector directing expression of the native bFGF) and NIH-IgBNM6-1 (transfected with the vector directing expression of the chimeric sp-bFGF), express the 18-kD immunoreactive species that comigrates with bFGF standard. Additional higher molecular weight species ($\sim$20, 24, 30, and 35 kD) are present in the cell line expressing the chimeric sp-bFGF. These species may represent the uncleaved form of sp-bFGF molecule as well as some bFGF molecules that were modified post-translationally upon entering the endoplasmic reticulum.

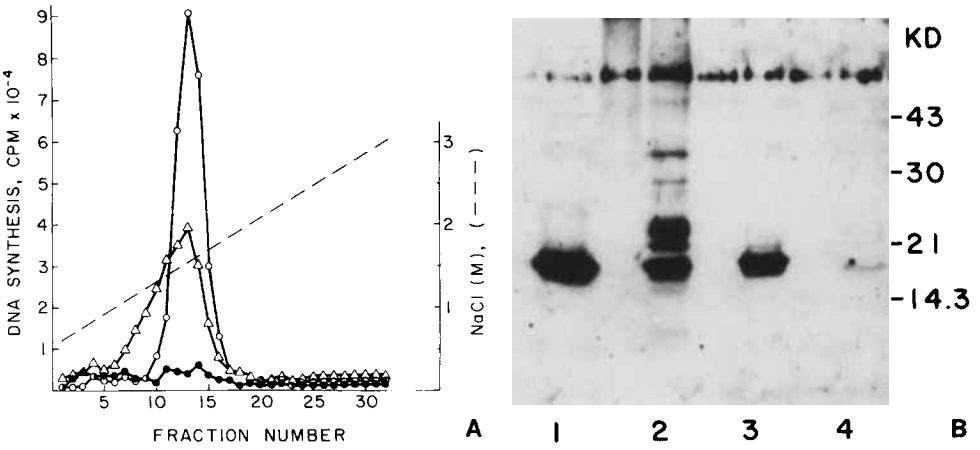

Fig. 1. **A:** Heparin-Sepharose Affinity chromatography elution profiles. Solid circles represent the control NIH-CONT cell lysate. Open circles represent the cell lysate of a bFGF-expressing cell line NIH-BNM7. Open triangles represent the cell lysate of a chimeric sp-bFGF-expressing cell line NIH-IgBNM6–1. **B:** Western blot analysis of the 1.5 M NaCl fractions from the above heparin-Sepharose column. **Lane 1:** Control recombinant bFGF. **Lane 2:** NIH-IgBNM6-I cell lysate. **Lane 3:** NIH-BNM7 cell lysate. **Lane 4:** NIH-CONT cell lysate.

No bFGF growth factor activity could be detected in the conditioned medium of any of the recombinant cell lines. Even when the media of the cell line expressing the chimeric sp-bFGF protein was conditioned in the presence of heparin (45 μg/ml), which stabilized free bFGF [6], no bFGF was detected in the medium. These results were confirmed using anti-bFGF amino-terminus peptide antibodies in an immunoprecipitation analysis (not shown).

Tumorigenicity of Cell Lines Expressing the Native or the Chimeric sp-bFGF

The tumorigenic potential of these and other cell lines was determined in both the immunocompetent syngeneic NIH/NSF and the athymic, immunodeficient nude mice. The results are summarized in Table I. The control cell lines were not tumorigenic in either the immunocompetent or the immunodeficient mouse type. However, biosynthesis of even very low levels of the chimeric sp-bFGF endows the cell with the ability to form tumors in 100% of mice of either type.

The response of the syngeneic mice to the cell lines elaborating the various levels of the native bFGF is different from that of the nude mice. These cell lines are not tumorigenic in immunocompetent mice even at levels as high as 4.6 units/10^4 cells, almost 10 times as much bFGF as is found in the endothelial cells. On the other hand, the tumors in nude mice do form with higher frequency when the cell lines produce elevated levels of the native bFGF. However, unlike the sp-bFGF tumors which appear at 100% frequency within 1–2 weeks of injection, these tumors have a late onset at around 5 weeks postinjection and occur with a frequency of about 50%.

Tumors Derive From the Injected NIH-3T3 Cells That Express bFGF

Neomycin-resistant cell lines were cultured from sp-bFGF tumors in both nude and syngeneic animal models and were analyzed for bFGF production using heparin affinity chromatography and immunoprecipitation. Figure 2 shows the heparin-Se-

TABLE I. Tumorigenicity of bFGF- and sp-bFGF-Expressing NIH-3T3 Cell Lines in the Syngeneic NIH/NSH Mice and the Immunodeficient NUDE/NUDE Mice

Cell lines injected	Units bFGF/ 10^4 cells	Tumors/ NSF mice	Time of appearance	Tumors/ NUDE mice	Time of appearance
NIH-CONT	0.06	0/12	0/4 mo[a]	0/6	0/3 mo[a]
NIH-BNMpc[c]	0.45	0/6	0/4 mo	0/6	0/3 mo
NIH-BNM6	0.6	0/6	0/3 mo	0/6	0/3 mo
NIH-BNM7	0.8	1/6	5 wk/3 mo	4/6	3 wk/3 mo
NIH-BNM46	1.4	0/3	0/5 mo	2/4	5 wk/3 mo
NIH-BNM48	1.4	0/3	0/5 mo	1/4	5 wk/3 mo
NIH-BNM39	3.8	0/3	0/5 mo	3/4	5 wk/3 mo
NIH-BNM35	4.6	0/3	0/5 mo	3/4	5 wk/3 mo
NIH-IgBNM6-1	0.8	38/38	1-2 wk	6/6	1-2 wk
NIH-IgBNM4-2	0.7	6/6	1-2 wk	4/4	1-2 wk
NIH-IgBNM29-1	0.7	6/6	1-2 wk	4/4	1-2 wk
NIH-EJ1	0.1	6/6	1-2 wk	3/3	1-2 wk
NIH-EJB10	2.2	6/6	1-2 wk	—	—
NIH-EJIgB13	0.8	3/3	1-2 wk	—	—

[a]0/4 mo means that no tumors in 4 months of observation.
[b]5 wk/3 mo means that the only tumors observed reached the size of ~0.5 cm^3 after about 5 weeks. The rest were tumor free for 3 months.
[c]NIH-BNMpc is a polyclonal neomycin-resistant cell line that expresses bFGF.

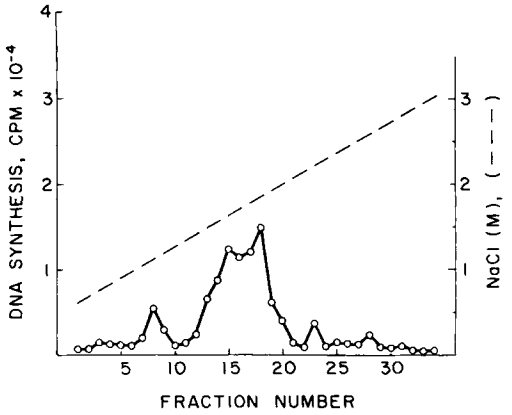

Fig. 2. Heparin-Sepharose affinity chromatography. Elution profile of a cell line derived from NIH-IgBNM6-1 tumor in an NIH/NSF mouse.

pharose elution profile of a cell line derived from a tumor that arose in an NIH/NSF mouse upon injection with the NIH-IgBNM6-1 cells. This neomycin-resistant cell line still expresses growth factor activity that elutes from heparin with 1.5 M NaCl. Also, a major 18-kD protein and several minor, higher molecular weight (~20 and 24 kD) proteins could be precipitated with the antisera raised against the amino-terminus peptide of bFGF (data not shown). These results indicate that the tumors did in fact arise from the injected, neomycin-resistant, chimeric sp-bFGF producing cell lines rather than from the host mouse cells.

Histological Analysis of the sp-bFGF Tumors

Histological analysis was performed on the sp-bFGF tumors from nude and immunocompetent syngeneic mice (Fig. 3A,B). Tumors in both animal types grow rapidly and are poorly differentiated. The tumors in the nude mice are far more vascularized than those from the same cell line growing in immunocompetent mice.

Specific Humoral Antibodies in Mice Bearing sp-bFGF Tumors

The immunocompetent NIH/NSF mice were checked for production of any specific antibodies that might be directed against bFGF or other novel antigens that may be present on the surface of the transformed cells. Control sera were obtained from mice injected with an equal number of the nontumorigenic parental NIH-3T3 cells.

The antibody fingerprint of sp-bFGF tumor-bearing sera is shown in Figure 4. While no proteins are immunoprecipitated by the control sera (Lane 1), a single strong 30-kD protein is specifically recognized by sera from two mice, each bearing a tumor that formed from an independently generated sp-bFGF- transformed cell line (Lanes 2, 3). This protein is uniquely recognized by sera from more than 40 mice injected with four independently isolated sp-bFGF clonal cell lines (data not shown). The antibody fingerprint of sp-bFGF tumors differs dramatically from the antibody fingerprint that forms in animals bearing tumors arising from *ras*-transformed NIH-3T3 cells. As seen in Lane 4, *ras* tumors also solicit a very specific antibody response in mice. This antibody fingerprint, as seen through the immunoprecipitating proteins, is also highly reproducible and was seen in more than ten mice bearing tumors formed from tumorigenic cells transformed with the *ras* oncogene.

To test for antigenic additivity of these tumors, cell lines stably expressing both sp-bFGF and *ras* were selected, injected into NIH/NSF mice, and the sera of the tumor-bearing animal were analyzed for presence of the *ras* and sp-bFGF antibody fingerprint in the IP assay. Indeed, in some cases, both sets of antibodies were found to be present in the same sera (Lane 6): the 30-kd protein characteristic of sp-bFGF tumors (as in Lanes 2, 3) and all the *ras* antibodies seen to be induced by *ras* tumors alone (as in Lane 4). A parallel set of cell lines expressing the native bFGF in addition to the *ras* proteins was also generated. Lane 5 represents the antibody fingerprint of one such *ras* tumor expressing the native bFGF.

The antigens to which the tumor-bearing mice developed antibodies are present not only on the tumor cells but are already expressed on the parental NIH-3T3 cells. In fact, additional data not shown here indicate that the 30-kD protein recognized by the sera of the NIH-IgBNM6–1 tumor-bearing mice is no more abundant in the immunizing NIH-IgBNM6–1 than it is in the nontumorigenic, parental NIH-3T3 cell line. In general, this observation is also true for most of the antigens recognized by the sera elicited by the *ras* tumors.

DISCUSSION

Work from a number of laboratories over the past years has indicated that autocrine growth stimulation can transform cells. This autocrine transformation requires the cellular expression of receptors capable of recognizing and responding to growth factors secreted by the cell itself [8–11].

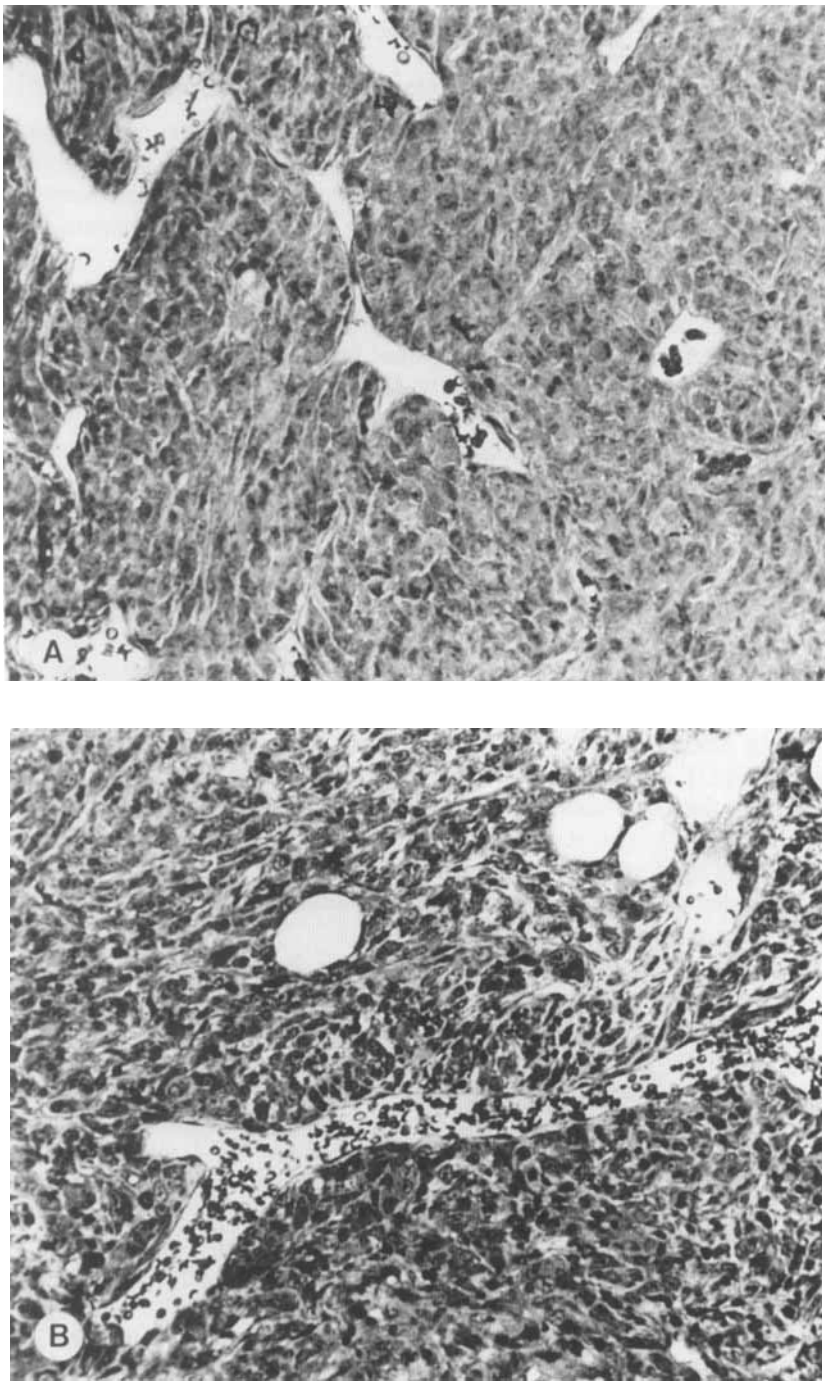

Fig. 3. Histology of NIH-IgBNM6-1 tumors. **A:** NUDE/NUDE mice. **B:** NIH-NSF mice.

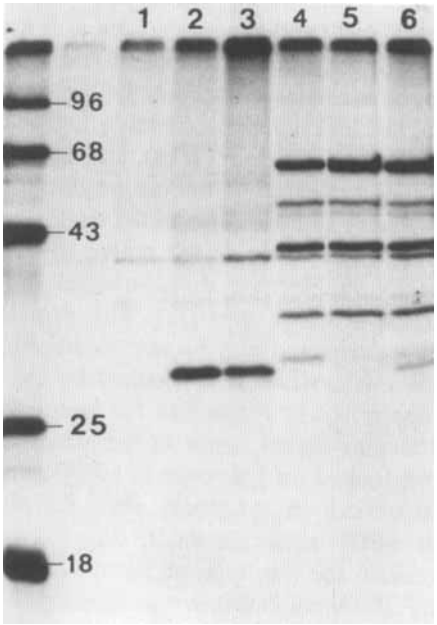

Fig. 4. Antibody fingerprinting of tumor-bearing sera. **Lane 1:** Control sera from a mouse injected with NIH-CONT cells. **Lanes 2, 3:** Sera from two mice injected with two independent sp-bFGF-producing clonal cell lines NIH-IgBNM6-1 and NIH-IgBNM29-1. **Lane 4:** Sera from an animal bearing an NIH-EJ1 tumor. **Lane 5:** Sera from an animal bearing tumor caused by an NIH-EJ cell line that also expresses the native bFGF (NIH-EJB10). **Lane 6:** Sera from an animal bearing a tumor caused by an NIH-EJ cell line also expressing the chimeric sp-bFGF (NIH-EJIgBI3). NIH-3T3 cell lysate was used as a substrate for this IP.

bFGF is a ubiquitous growth factor that is mitogenic for a wide variety of cell types, some of which also produce this factor. Yet, mere production of bFGF does not cause growth or transformation of such bFGF responsive cells. For example, bovine aortic endothelial and human umbilical vein endothelial cells synthesize high levels of intracellular bFGF [14,17], yet they require exogenous bFGF in order to proliferate. We have recently shown that bFGF is capable of tumorigenic transformation of NIH-3T3 fibroblasts only when a signal peptide is fused to bFGF. We speculate that in these cells, bFGF is localized in a subcellular compartment in which it has access to its cognate receptor.

Since bFGF is an angiogenic factor [18], the tumors induced by its autocrine action might be expected to be typified by characteristics such as unusually rich vascularization. The sp-bFGF tumors in nude mice indeed have prolific vasculature. In contrast, the tumors of the same origin in NIH/NSF mice are quite similar to *ras* induced sarcomas, and have inconspicuous vasculature. The reason for this is unclear. Perhaps some bFGF is secreted from the cells or simply leaks out of the necrosing tumor tissue. While the nude mice may respond to the angiogenic effect of externalized bFGF, the syngeneic mice may mount an immune defense against this new protein and neutralize it, thereby developing less vascularized tumors. However none of the tumor sera tested had detectable antibody titer to bFGF, so that the leakage of

bFGF into the extracellular space in the tumor is probably not great. Thus, this discrepant vascularization of nude and syngeneic tumors is not explained.

In striking contrast with the immunocompetent mice, the nude mice do develop tumors from cells that synthesize large amounts of the native bFGF. However, in these cells the required bFGF levels are considerably higher (2–8 times), the tumors take much longer to form (5 weeks vs. 1–2 weeks), and they arise at a lower frequency (50% ± 25% vs. 100%) as compared to tumors produced by sp-bFGF-expressing cells. Again it is not clear why tumors form more readily in the nude mice. These cell lines that express very high levels of the native bFGF, though neither focus-forming nor morphologically transformed, do have lower serum requirements and do grow to higher saturation densities *in vitro* [12]. This growth may be dependent on leakage of bFGF from dying cells and may be responsible for tumor formation in the immunoincompetent nude mice, while it is blocked by the immune system in the syngeneic mice. If leakage is solely resposible for tumor formation in nude mice, then, in addition to the vascular tissues, some of the tumor mass may be made up of host cells. We have not yet looked for host cells in bFGF tumors in nude mice.

Although sp-bFGF tumors in syngeneic mice do not cause an identifiable antibody response to the bFGF molecule itself, they do evoke a typical humoral response that is characteristic for this type of tumor and is the same for all mice regardless of which sp-bFGF clonal cell line was injected. The mice develop a high titer of humoral antibodies directed specifically against a 30-kD protein of as yet undetermined subcellular localization. This 30-kD protein is not a tumor-specific antigen since it is found in equal abundance in the nontransformed parental cell line. This antigen may be presented in a new, immunogenic way in the sp-bFGF producing cells as compared to the parental NIH-3T3 cells. Although unlikely, it is also conceivable that the tumor formation removes immune self-tolerance for this particular antigen. Similarly, antibodies against a different set of self-antigens arise in mice injected with *ras* tumors. At least is some cases, both sets of antibodies are developed in response to sp-bFGF-producing *ras* tumors.

The antibody fingerprint as visualized in our IP assay enbles us to determine the transforming lesion within 1 week of tumor detection. Perhaps similar analysis of sera collected from patients with *hst* tumors (Kaposi sarcoma, stomach cancers) and others may detect an antibody fingerprint that will aid in tumor diagnosis and treatment.

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