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Immunology

Protection of mice against tumor growth by immunization with an oncogene-encoded growth factor

(tumor prevention/K-*fgf* oncogene/recombinant proteins)

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ABSTRACT **The K-*fgf*/*hst* oncogene encodes a growth factor of the fibroblast growth factor (FGF) family that is secreted and transforms cells through a mechanism of autocrine cell proliferation. K-*fgf*-transformed cells are highly tumorigenic in immunocompetent allogeneic and syngeneic animals. BALB/c mice were immunized with a bacterial fusion protein consisting of a portion of the MS2 polymerase and of the human K-FGF precursor lacking only the first 4 amino acids or with a recombinant protein corresponding to the mature, secreted form of K-FGF (176 amino acids). They were then challenged with syngeneic K-*fgf*- or H-*ras*-transformed cells. Vaccinated animals exhibited a significant degree of protection against tumor induction, which was specific for K-*fgf*-transformed cells and correlated with the ability of the immunized mice to produce high titers of anti-K-FGF antibodies. Thus immunization with a single oncogene product can protect animals against tumor cells expressing this oncogene.**

Tumors often express specific transplantation antigens, which are located on the cell surface and thus accessible to immune cells and antibodies (1). A number of studies have been directed to the identification of tumor-associated markers that could be used as a target for either active or passive immunotherapy (1–4). Most of these studies however have utilized antibodies generated against undefined structures on the malignant cell surface (1–4). A more effective strategy may be to direct passive or active immunization against a defined cell surface molecule whose expression is essential for the cancerous cell growth (5–9). The products of activated cellular oncogenes, if exposed on the surface of tumor cells, could provide an appropriate target for such immune therapy.

The K-*fgf*/*hst* oncogene encodes a growth factor of the fibroblast growth factor (FGF) family and transforms NIH 3T3 and BALB/c 3T3 cells with high efficiency (10, 11). The activation of this oncogene results from overexpression (12, 13), and thus the protein product of the oncogene and that of its corresponding protooncogene are identical. The K-FGF is a glycoprotein that is efficiently secreted (13) and appears to induce the transformed phenotype by binding to the cell surface receptors and creating an autocrine growth loop (13). Thus K-*fgf*-transformed cells should be an appropriate model system for the study of targeted immunotherapy. Cellular and humoral immunity could be effective against such transformed cells; neutralizing antibodies could inactivate the growth factor extracellularly and thus interrupt the autocrine growth loop. Cytotoxic T cells could also be targeted against the tumor cells that expose the growth factor or fragments of it on their surface, and antibody-mediated cell lysis could cooperate in the selective destruction of the K-*fgf*-transformed cells. Here we report that mice immunized with human K-FGF exhibit a substantial degree of protection against tumors produced by the injection of K-*fgf*-transformed cells.

MATERIALS AND METHODS

Cell Culture and Transfection. BALB/c 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Cells (10^6 per 100-mm dish) were transfected by the calcium phosphate precipitation technique with 0.5 μg of p9BKS3A or pTB-1 plasmid plus 12 μg of NIH 3T3 carrier DNA. p9KS3A contains the human K-*fgf* cDNA inserted at the *Eco*RI site of the 91023B expression vector (10). The pTB-1 plasmid carries the *Bam*HI–*Hin*dIII fragment of an activated human *HRAS* (14). BALB K-*fgf* and BALB *ras* cell lines were derived from individual foci, after recloning in soft agar.

Protein Purification. The MS2/K-FGF fusion protein was purified from the *Escherichia coli* strain K12ΔH1Δtrp transformed with the recombinant plasmid pEX34KS3A, which contains the 0.9-kilobase (kb) *Sma* I-*Eco*RI fragment of the human K-*fgf* cDNA (10). The fusion protein consists of the first 99 amino acids of the MS2 polymerase, followed in frame by the K-FGF precursor protein lacking the first 4 amino acids. Purification of the protein was performed as described by Strebel *et al.* (15). The 7 M urea extract was extensively dialyzed against 50 mM Tris·HCl, pH 7.9/5 mM EDTA/1 mM dithiothreitol and then centrifuged at 15,000 rpm for 40 min. After determination of the protein concentration, the supernatant was used to immunize mice.

The recombinant K-FGF protein was purified from an *E. coli* culture transformed with the plasmid pBR/KSF(A), which expresses the region of K-*fgf* cDNA coding for the 176-amino-acid secreted K-FGF protein under the control of the λ P_L promoter. The pellet obtained after cell lysis was extracted with 1 M NaCl, and following a series of precipitation steps the soluble protein was passed through a Q Toyo-Pearl column and then loaded on heparin-Sepharose. The K-FGF protein was eluted at 1.1 M NaCl (D. Rogers and N. Wolfman, personal communication).

Antibody Measurements. Serum antibody response to K-FGF was detected using a solid-phase ELISA. Immulon II microtiter plates were coated with 10 ng of recombinant mature K-FGF and blocked with 0.1% Tween 20 containing 10 mg of bovine serum albumin per ml. Serum samples were diluted with phosphate-buffered saline and added to the wells. After washing, alkaline phosphatase-coupled anti-mouse immunoglobulin [goat anti-mouse IgG (heavy and light chains), diluted 1:300] was added to each well, incubated, and

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Abbreviation: FGF, fibroblast growth factor.
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washed. The bound enzyme was detected using the ELISA amplification system supplied by Bethesda Research Laboratories (16), and the OD at 495 nm was read using an automated ELISA reader.

The presence of anti-K-FGF neutralizing antibodies was tested by assaying the ability of the mice serum to induce reversion of the transformed morphology of BALB K-*fgf* cells and to inhibit stimulation of DNA synthesis by K-FGF in quiescent NIH 3T3 cells, using as a control a rabbit anti-K-FGF serum that shows neutralizing activity up to 1:1000 dilution.

RESULTS

NIH 3T3 cells transformed by the human K-*fgf* oncogene are highly tumorigenic in nude mice. When injected at high doses, they can also form tumors in immunocompetent BALB/c mice (unpublished results). We considered it important to attempt immunization in a syngeneic system, and therefore we used a clone of K-*fgf*-transformed BALB/c 3T3 cells and BALB/c mice for all experiments. We first tested the tumorigenic potential of the BALB K-*fgf* cell line. These cells were isolated after transfection with the p9BKS3A plasmid (10), which carries the K-*fgf* human cDNA under the control of the adenovirus major late promoter. As few as 2×10^4 cells uniformly produced progressively growing tumors when injected into 9-week-old BALB/c mice. We used as an antigen for the immunization an MS2/K-FGF bacterial fusion protein that consists of the first 99 amino acids of the MS2 polymerase, followed in frame by the entire K-FGF precursor protein lacking only the first 4 amino acids (10). The protein was partially purified as described in *Materials and Methods*. It is substantially denatured but has good immunogenic activity in rabbits and shows biological activity in assays of stimulation of proliferation of NIH 3T3 cells (10).

In a first series of experiments, 4-week-old BALB/c mice were immunized with 30 μg of the partially purified K-FGF fusion protein. This extract contained about 20 μg of K-FGF and 10 μg of contaminating bacterial proteins. Control mice were injected with ≈10 μg of proteins prepared from an equivalent extract of bacteria transformed by the plasmid vector alone. The immunization protocol consisted of four injections at 10-day intervals. Ten days after the last booster injection, the mice were bled, and 2 days later the mice were challenged with 10^5 BALB K-*fgf* cells or, as a control for the specificity of the vaccination procedure, BALB/c cells transformed by the *HRAS* oncogene.

The results of this experiment, shown in Table 1, indicate that after 70 days, all of the mice immunized with the control protein and challenged with BALB K-*fgf* cells had developed tumors, but 42% of the mice immunized with the K-FGF fusion protein were tumor free. In the group challenged with the H-*ras*-transformed BALB/c cells the immunization did not result in any protection against the tumor. In order to correlate the inhibition of tumor growth with the immune response to the K-FGF protein, we tested the production of antibodies using an ELISA. This analysis showed that the sera from all of the animals that were protected against the tumor were positive at a 1:800 dilution or higher. On the other hand, animals that succumbed to tumors had antibody titers lower than 1:200. Thus these results suggested that mice that mounted a more efficient immune response to the vaccine were protected against the tumors, whereas inefficient responders were not.

In an attempt to improve the protection against the tumor, we performed another experiment with a slightly modified immunization protocol. Two groups of 24 mice were subjected to a series of five injections with 20 μg of K-FGF recombinant fusion protein or equivalent amounts of control protein. One week after the last injection, the animals were challenged with either 10^5 or 5×10^4 BALB K-*fgf*-transformed cells. As shown in Table 1, 70 days later about 60% of the mice in each of the groups immunized with the K-FGF protein remained tumor free, whereas 100% of the control animals had developed tumors. In addition, those vaccinated animals that developed tumors did so with a substantial delay (Fig. 1). The anti-K-FGF antibody content of the serum was again measured by ELISA, using as an antigen a bacterial protein corresponding to the mature, secreted form of K-FGF (176 amino acids) (13) and thus lacking the MS2 polymerase sequences (see *Materials and*

Table 1. Effect of immunization against a K-FGF fusion protein on the tumorigenicity of transformed BALB/c 3T3 cells in syngeneic mice

Group	Immunization	Tumor cell challenge*	Tumor-free animals†
		Experiment 1	
A	K-FGF	BALB K-*fgf*	5/12
B	Control	BALB K-*fgf*	0/12
C	K-FGF	BALB *ras*‡	0/12
D	Control	BALB *ras*‡	0/12
		Experiment 2	
A	K-FGF	BALB K-*fgf*	7/11
B	Control	BALB K-*fgf*	0/11
C	K-FGF	BALB K-*fgf*§	7/12
D	Control	BALB K-*fgf*§	0/12

Four-week-old BALB/c mice were injected i.p. with 20 μg of the MS2/K-FGF fusion protein in complete Freund's adjuvant or with an equivalent amount of extract derived from bacteria expressing the plasmid vector only. This procedure was repeated three times (experiment 1) or four times (experiment 2) with 10-day intervals using incomplete Freund's adjuvant. Twelve days (experiment 1) or 7 days (experiment 2) after the last immunization the animals were injected s.c. with the transformed BALB/c 3T3 cells. Animals were inspected weekly for the appearance of palpable tumors.

*All mice were challenged with 1×10^5 cells unless indicated otherwise.

†At 70 days after tumor cell challenge.

‡Isolated after transfection with the pTB-1 plasmid (15), which carries an activated human *HRAS* oncogene.

§Mice were challenged with 5×10^4 cells.

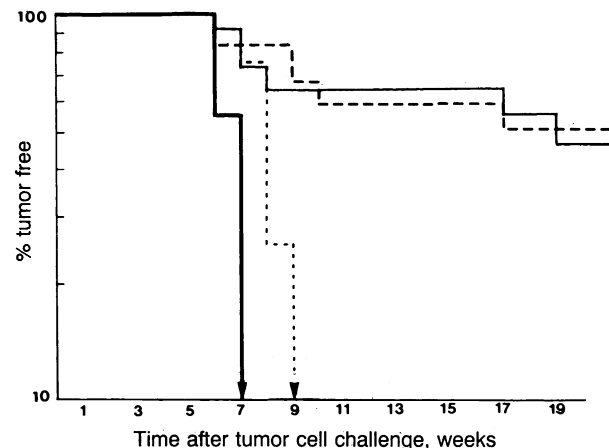

FIG. 1. Protection of immunized mice against BALB K-*fgf*-transformed cells. BALB/c mice immunized with the K-FGF fusion protein or control animals were challenged with either 1×10^5 or 5×10^4 K-*fgf*-transformed BALB/c 3T3 cells injected s.c. Animals were checked every week for the presence of tumors at the injection site. Tumors were scored when palpable (diameter > 2 mm). Solid lines indicate 1×10^5 cells injected: control mice (▬), vaccinated mice (—). Broken lines indicate 5×10^4 cells injected: control mice (--), vaccinated mice (—). The arrows indicate that no animals were tumor free at the time shown. The data are from the experiment shown as experiment 2 of Table 1.

Methods). The recombinant K-FGF polypeptide is essentially purified to homogeneity and thus does not contain any detectable *E. coli* proteins. As indicated in the first two lines of Fig. 2, virtually all immunized mice make antibodies against the K-FGF protein. Immunized animals that remained tumor free maintained antibody titers that were not different than those found prior to tumor challenge. Mice that developed tumors had significantly lower titers (Fig. 2). Challenge with K-*fgf*-transformed cells itself was sufficient to elicit antibody production (Fig. 2, last line) but did not protect the animals. It should be noted that three of the immunized animals developed tumors after a very long latency period (Fig. 1). Ten weeks after tumor challenge, these three mice had low antibody titers, comparable to those found in unimmunized animals. Thus the antibody titer against K-FGF correlated with the response of each animal to the challenge with the transformed cells, confirming the observations made during the first experiment.

The ability of the serum obtained from tumor-free animals to neutralize K-FGF was also measured. These sera had no detectable blocking activity for exogenous K-FGF and did not affect the growth of the tumor cells *in vitro*. Because of the small quantity of serum available from each mouse, we cannot however exclude the presence of low titers of neutralizing antibodies.

Although the degree of tumor protection observed in our immunization experiments was high, a significant proportion of the vaccinated animals still developed tumors. The fact that protection correlated with the production of high titers of antibodies seemed to indicate that variations in the individual immune response played a major role (17). We considered the possibility that the nature of the immunogen used, a bacterial fusion protein that was substantially denatured and unlikely to be in the natural antigenic configuration (10), could have been responsible for this high level of individual variations. To test this hypothesis, we immunized animals with purified, recombinantly produced K-FGF protein. This non-glycosylated protein, which encompasses the secreted form of K-FGF, shows very high biological activity in assays of DNA synthesis induction in serum-starved NIH 3T3 cells. Thirteen 4-week-old BALB/c mice were injected i.p. with 4 μg of K-FGF in complete Freund's adjuvant. Four booster injections were administered at 10-day intervals. As a control, a group of 10 mice was subjected to the same injection protocol with saline and Freund's adjuvant only. As shown in Fig. 3, the occurrence of tumors in the group of K-FGF-immunized animals was significantly delayed as compared to the control mice, and 60 days after tumor cell challenge, when all of the control mice had already developed large tumors, 4 of the 13 K-FGF-immunized mice remained tumor free. Since these animals had been immunized with a native K-FGF, it appears that the configuration of the immunogen used was not responsible for the fact that a substantial proportion of vaccinated animals was not protected.

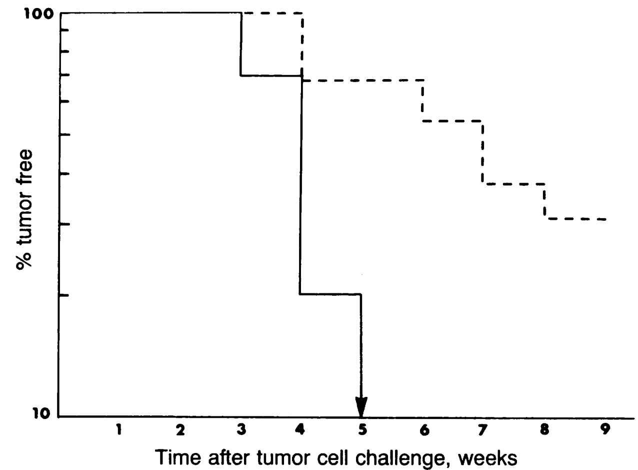

FIG. 3. Protection against BALB K-*fgf*-transformed cells of mice immunized with the mature recombinant protein. BALB/c mice immunized as described or control animals were challenged with 10^5 BALB K-*fgf* cells. Tumors were scored when palpable (diameter > 2 mm). —, Control mice; --, vaccinated mice. The arrow indicates that no mice were tumor free.

DISCUSSION

Taken together, our results show that it is possible to obtain a significant (>50%) tumor protection in BALB/c mice by vaccination with a single oncogene product that is expressed and secreted by the injected tumor cells. Protection is specific for cells transformed by the K-*fgf* oncogene and thus does not result from a general boosting of immune response. Our results are similar to those reported by Bernards *et al.* (7) with the *neu* oncogene and by Lathe *et al.* (8), using the polyoma virus-transforming proteins, but they differ in one important aspect. Those authors used a recombinant vaccinia virus for immunization, which would express the desired antigens only after infection of susceptible cells, whereas we immunized animals with a soluble protein. Thus, the type of immune response elicited by these procedures would be expected to differ. In addition, the results with the *neu*

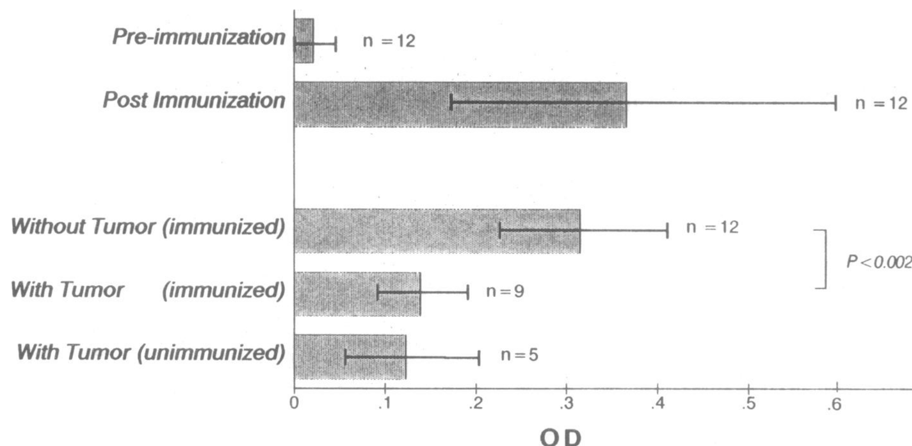

FIG. 2. ELISA of the anti-K-FGF activity in the serum of mice immunized with MS2/K-FGF fusion protein. The animals were immunized as described in the text. The postimmunization samples were obtained 2 days after the last injection of the K-FGF fusion protein. The remainder of the animals were bled either at the time of appearance of a palpable tumor or 70 days after challenge. The assay was performed as described in the text. OD is proportional to antibody content.

oncogene were obtained in a nonsyngeneic system, in which tumors eventually regress spontaneously.

A route of immunization similar to the one we used was attempted by Ramquist *et al.* (18), using polyoma virus antigens. It is not clear, however, whether these antigens are expressed on the surface of polyoma virus-transformed cells, and clearly they are not secreted. Thus the effector mechanisms involved in protection may be different.

In this regard we cannot assess whether the immune response conferring protection in our experiments is humoral or cellular. Almost all immunizations lead to the development of cellular and humoral immunity. Resistance to solid tumors is usually better correlated with cellular immunity than antibody titer, but it has been well documented that antibodies can destroy grafts consisting of cell suspensions (19, 20). Despite the correlation between the mounting of an efficient humoral immune response and survival observed in our experiments, the possibility that cellular immunity plays an important role cannot be ruled out. The antibodies detected in the serum of immunized mice are mostly nonneutralizing, a finding that would tend to rule out the hypothesis that tumor growth is suppressed by neutralization of the growth factor itself. However, the presence of low-titer neutralizing activity or of locally produced antibodies cannot be ruled out. We also measured the presence of cytotoxic antibodies specific for K-*fgf*-transformed cells. The antibodies detected in the serum of immunized mice had cytotoxic activity in the presence of complement, but unimmunized, tumor-bearing animals developed similar levels of cytotoxic antibodies (data not shown).

Finally, it has to be said that the protein used for immunization as well as the oncogene responsible for cell transformation were of human origin, and it is quite possible that mice could not have mounted an effective immune response against cells transformed by the murine K-*fgf* oncogene. It is therefore possible that the success of our experiment depended on the xenogeneic origin of the antigen used to induce an immune response. Preliminary results indicate that immunization with a human K-FGF protein does not significantly inhibit tumor growth of BALB/c cells transformed by a murine K-*fgf* oncogene. In spite of the very high degree of homology (about 90%) between the human and murine proteins (21) and the likelihood of significant structural similarity, the major antigenic determinants are probably quite different.

Immunization with the mouse K-FGF protein and challenge with BALB/c cells transformed by the murine K-*fgf* will allow us to determine whether it is possible to observe tumor protection in a totally syngeneic system.

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1. Schreiber, H., Ward, P. L., Rowley, D. A. & Stauss, H. J. (1988) *Annu. Rev. Immunol.* **6,** 465–483.
2. Boss, B. D., Langman, R., Trowbridge, I. & Dulbecco, R. (1983) *Monoclonal Antibodies and Cancer* (Academic, New York).
3. Reisfeld, R. A. & Sell, S. (1985) *Monoclonal Antibodies and Cancer Therapy* (Liss, New York).
4. Herlyn M. & Koprowski, H. (1988) *Annu. Rev. Immunol.* **6,** 283–308.
5. Cuttitta, F., Carney, D. N., Mulshine, J., Moody, T. W., Fedorko, J., Fischler, A. & Minna, J. D. (1985) *Nature (London)* **316,** 823–826.
6. Drebin, J. A., Link, V. C., Weinberg, R. A. & Greene, M. I. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9129–9133.
7. Bernards, R., Destree, A., McKenzie, S., Gordon, E., Weinberg, R. A. & Panicali, D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6854–6858.
8. Lathe, R., Kieny, M. P., Gerlinger, R., Clertant, P., Guizani, I., Cuzin, F. & Chambon, P. (1987) *Nature (London)* **326,** 878–880.
9. Drebin, J. A., Link, V. C. & Greene, M. I. (1988) *Oncogene* **2,** 273–277.
10. Delli-Bovi, P., Curatola, A. M., Kern, F. G., Greco, A., Ittmann, M. & Basilico, C. (1987) *Cell* **50,** 729–737.
11. Taira, M., Yoshida, T., Miyagawa, K., Sakamoto, H., Terada, M. & Sugimura, T. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2980–2984.
12. Yoshida, T., Miyagawa, K., Odagiri, H., Sakamoto, H., Little, P. F. R., Terada, M. & Sugimura, T. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7305–7309.
13. Delli-Bovi, P., Curatola, A. M., Newman, K. M., Sato, Y., Moscatelli, D., Hewick, R. M., Rifkin, D. B. & Basilico, C. (1988) *Mol. Cell. Biol.* **8,** 2933–2941.
14. Goldfarb, M., Shimizu, K., Perucho, M. & Wigler, M. (1982) *Nature (London)* **296,** 404–409.
15. Strebel, K., Beck, E., Strohmaier, K. & Shaller, H. (1986) *J. Virol.* **57,** 983–991.
16. Stanley, C. J., Johannsson, A. & Self, C. H. (1985) *J. Immunol. Methods* **83,** 89–95.
17. Staudt, L. M. & Gerhard, W. (1983) *J. Exp. Med.* **157,** 687–704.
18. Ramquist, T., Pallas, D. O., DeAnde, J., Ahrlund-Richter, L., Reinholdsson, G. & Roberts, T. M. (1988) *Int. J. Cancer* **42,** 123–128.
19. Russell, P. S. & Monaco, A. P. (1964) *N. Engl. J. Med.* **271,** 502–510.
20. Lafferty, K. J., Prowse, S. J., Simeonovic, C. J. & Warren, H. S. (1983) *Annu. Rev. Immunol.* **1,** 143–173.
21. Brookes, S., Smith, R., Thurlow, J., Dickson, C. & Peters, G. (1989) *Nucleic Acids Res.* **17,** 4037–4045.