

# Identification of Fibroblast Growth Factor-5 as an Overexpressed Antigen in Multiple Human Adenocarcinomas

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## ABSTRACT

**Methodology for identifying tumor-associated antigens recognized by T cells has been successfully used to clone antigens from melanoma cells. Similar efforts for nonmelanoma tumors have had limited success with few antigens identified. To identify potentially relevant tumor-associated antigens expressed in renal cell carcinoma cell lines, a tumor-specific CTL clone was established from tumor-infiltrating lymphocytes from a regressing pulmonary lesion. This CTL recognized nonmutated fibroblast growth factor-5 (FGF-5). Quantitative real-time reverse transcription PCR revealed that FGF-5 was overexpressed in the majority of renal cell carcinomas, as well as in some prostate carcinoma and breast carcinoma lines. FGF-5 expression by quantitative real-time reverse transcription PCR in normal tissues was below the recognition threshold for this CTL. As a normal protein with significant overexpression by multiple adenocarcinomas and little normal tissue expression, FGF-5 represents an immunotherapy target with potential utility against a broad array of nonmelanoma cancers.**

## INTRODUCTION

Malignant melanoma and RCC<sup>3</sup> are two tumors for which immunotherapy has been shown to be beneficial (1–3). Identification of melanoma-associated antigens has been especially successful and opened new possibilities for the immunotherapy of patients with this cancer. At the Surgery Branch of the NCI, addition of a peptide vaccine from a melanoma-associated antigen to high-dose IL-2 therapy resulted in an enhanced response rate (42% compared with 17% for a nonrandomized control population; Ref. 2). However, far less progress has been made with regards to identification of RCC-associated antigens. Several molecules have been reported as RCC-associated antigens recognized by T cells. Two of these, mutated HLA-A2 (4) and hsp70-2 (5), are patient-specific mutated antigens, whereas the other three are nonmutated, potentially shared antigens. RAGE-1 was the first antigen to be identified as a RCC-associated nonmutated antigen (6). However, the frequency of RAGE-1 expression in RCC was reported to be as low as 1 in 57, limiting its clinical utility. A recent report by Neumann *et al.* (7) showed 8 of 39 RCC samples (21%) to be positive by RT-PCR analysis, but correlation of RT-PCR and immunological detection was not investigated. A second nonmutated RCC antigen is iCE. The identified epitope in this antigen is generated by a non-AUG-defined alternative ORF of the gene (8). Because iCE mRNA is expressed in normal organs such as liver, intestine, kidney, and heart, clinical utility will depend on demonstrating that translation of the non-AUG-defined alternative ORF transla-

tion is tumor specific and that this phenomenon occurs in other iCE-expressing tumors. The third nonmutated antigen is G250 (9). This antigen is expressed in 85% of RCCs and not in normal kidney. However, it is expressed in some gastric mucosal cells and cells in the bile ducts. Recognition of G250 in such normal cells has not been investigated. Currently, the question of whether tumor antigen vaccines can improve the response rate of RCC to IL-2 is unanswered and awaits the identification of suitable antigens. In addition, identification of shared T-cell antigens from more common tumors such as breast and prostate cancer remains a major goal in immunotherapy.

This study describes the establishment of a tumor-reactive TIL clone from a regressing lung metastasis in a patient showing a mixed spontaneous regression of metastatic RCC. The clone was found to recognize FGF-5 by expression cloning. An analysis of normal and malignant cell lines supports the applicability of this antigen in vaccination against not only RCC but also some prostate and breast cancers.

## MATERIALS AND METHODS

**Antibodies.** W6/32 (anti-pan class I MHC; Ref. 10), MA2.1 (anti-HLA-A2; Ref. 11), OKT3 (anti-CD3), and GAPA3 (anti-HLA-A3; Ref. 10) hybridomas were purchased from ATCC (Rockville, MD), and antibodies were purified by Lofstrand Labs (Gaithersburg, MD). B1-23-2 (anti-HLA-B/C; Ref. 10) was a kind gift of Dr. Paul Robbins (NCI, Bethesda, MD).

**TIL and Cell Lines.** The surgically resected remnant of a spontaneously regressing metastatic lung lesion was enzymatically digested (0.1% collagenase type IV, 30u/ml DNase type IV, and 0.01% hyaluronidase type V; Sigma Chemical Co., St. Louis, MO) at room temperature for 3 h, filtered through 100- $\mu$ m nylon mesh, and separated by density gradient using Lymphocyte Separation Medium (Organon Teknica, Durham, NC). The lymphocyte-containing interface was washed and cultured in RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10% fresh human serum (Biochemed Pharmaceuticals, Winchester, VA), 0.3% glutamine (Biofluids), 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ g/ml gentamicin, 0.5  $\mu$ g/ml fungizone (Biofluids), and 6000 IU/ml of recombinant human IL-2 (Chiron Corp., Emoryville, CA). TIL cultures were initiated in 24-well plates at  $1 \times 10^6$  cells/well and stimulated every 2 weeks with an irradiated (100 Gy) autologous RCC cell line established from the primary tumor. After 3 months of stimulation, the bulk culture was subjected to limiting dilution.  $\gamma$ -irradiated (120 Gy) autologous EBV-B cells ( $1 \times 10^4$  cells/well) and  $\gamma$ -irradiated (40 Gy) allogeneic peripheral blood mononuclear cells ( $5 \times 10^4$  cells/well) were used as feeder cells. Anti-CD3 mAb (OKT3) was added at the final concentration of 30 ng/ml on day 0, and human IL-2 was added at the final concentration of 240 IU/ml on day 1. T cells cultured in bulk were plated at 1 and 5 cells/well, and clones/clonoids were assayed for tumor recognition on day 14. Selected clones/clonoids were expanded by using the method described by Walter *et al.* (12). Briefly, on day 0,  $0.5\text{--}5 \times 10^5$  T cells were cocultured with irradiated (40 Gy) peripheral blood mononuclear cells ( $2.5 \times 10^7$  cells) and irradiated (120 Gy) autologous EBV-B cells ( $2.5 \times 10^6$  cells) in 25 ml of RPMI 1640 medium containing 10% fresh human serum, 30 ng/ml OKT3 mAb. On day 1, human IL-2 was added to the final concentration of 240 IU/ml. On day 5 and 8, 20 ml of the media was replaced with fresh media containing 240 IU/ml IL-2. Expanded cells were harvested around day 12 and cryopreserved. This expansion was repeated twice to obtain enough numbers of T cells for the cDNA library screening. An autologous EBV-B cell line was established with virus-containing supernatant from cell line B95-9 (ATCC). An autologous fibroblast strain was established by infecting a 1-week-old cultured tumor sample with a

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<sup>3</sup> The abbreviations used are: RCC, renal cell carcinoma; IL, interleukin; RT-PCR, reverse transcription-PCR; iCE, intestinal carboxyl esterase; ORF, open reading frame; TIL, tumor-infiltrating lymphocyte; FGF-5, fibroblast growth factor-5; ATCC, American Type Culture Collection; mAb, monoclonal antibody; NCI, National Cancer Institute; q-RT-PCR, quantitative real-time RT-PCR.

retrovirus that encoded the papilloma virus E6/E7 proteins and performing limiting dilution. The HLA-A2-restricted CTL control clone that was used in the restriction element analysis was isolated by limiting dilution of a mixed lymphocyte tumor culture from an HLA-A2 patient with renal cell carcinoma. This CTL recognizes HLA-A2-expressing RCCs, and the activity is blockable by anti-HLA-A2 mAb, MA2.1. RCC cell lines UOK125, UOK127, UOK130, UOK131, UOK150, and UOK 171 were kind gifts of Dr. W. Marston Linehan (NCI). Lung cancer cell lines SKGT2, SKGT4, and SKGT5 and esophageal cancer cell line HCE-4 (13) were a kind gift of Dr. David Schrum (NCI). 1570 RCC, 1581 RCC, 1645 RCC, 1764 RCC, CY13, 501 mel, 526 mel, 586 mel, 624.38 mel, 888 mel, 1088 mel, 1199 mel, and 1479 mel were established from surgical samples in our laboratory. SW480, PC-3, and DU 145 were purchased from ATCC. TSU-PR1 (14) was a kind gift of Dr. Suzanne Topalian (NCI). To introduce HLA-A3 into non-HLA-A3-expressing cells, the *HLA-A3* gene was cloned from autologous 1764 RCC by RT-PCR, sequenced, and subcloned into the retroviral vector pRx-IRES-Bsr (15). Vesicular stomatitis virus G protein-pseudotyped retrovirus was prepared by transiently transfecting the 293 GP cell line as described (16). Non-HLA-A3 tumor cell lines were infected with filtered supernatant in the presence of 8 µg/ml of Polybrene. Infection efficiencies were typically 70–100% by flow cytometric analysis, and the cell lines were followed by further selection with 5 µg/ml of blasticidin S (Calbiochem, San Diego, CA).

**CTL Recognition Assay.** Cultured tumor lines (5–10 × 10<sup>4</sup>) or transduced COS cells were used as target cells. In the latter, 100 ng of plasmid were used to transfect 5 × 10<sup>4</sup> COS-A3 cells/well using 1 µl of Lipofectamine (Life Technologies, Inc., Rockville, MD) in 96-well plates. After an overnight culture, CTL clone 2 was added at 20,000 cells/well and incubated an additional 20 h, and the supernatant was assayed for IFN-γ concentration by ELISA (Endogen, Woburn, MA).

**MHC Blocking Experiments.** Autologous RCC cells (5 × 10<sup>4</sup> cells in 100 µl) were incubated with each blocking mAb at the concentration of 20 µg/ml. After a 30-min incubation at room temperature, T cells (10,000 cells/well) were added. Then, after 20 h of culture at 37°C, the supernatant was assayed for IFN-γ concentration by ELISA.

**Construction of the cDNA Library and cDNA Library Screening.** Poly (A)<sup>+</sup> RNA was prepared from the autologous renal cell carcinoma cell line using a mRNA isolation system (Fast Track kit 2.0; Invitrogen, Carlsbad, CA). cDNA was prepared with the Superscript plasmid system (Life Technologies, Inc.) and ligated into the eukaryotic plasmid expression vector pME18S (a kind gift of Dr. Atsushi Miyajima, University of Tokyo, Tokyo, Japan). After electroporation and titration, a cDNA library was prepared in pools by inoculating approximately 100 bacterial clones/well in 1 ml of Luria Broth/well and purifying plasmid using the Qiaprep 96 turbo system (Qiagen, Valencia, CA).

**q-RT-PCR.** Quantitative analysis of FGF-5 mRNA expression was done using the ABI prism 5500 or 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA) as described (17). Thermal cycler parameters were: 2 min at

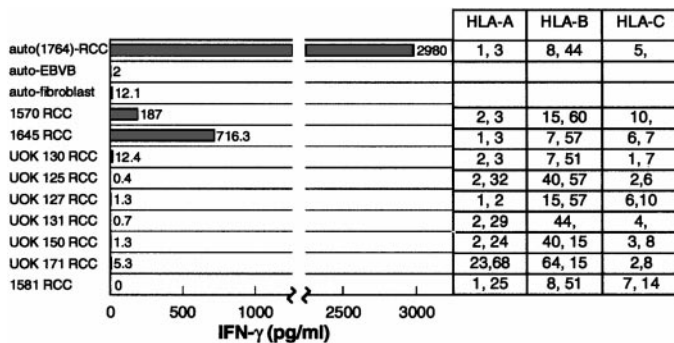


Fig. 1. Analysis of the reactivity of CTL clone 2 against a panel of tumor cell lines. Reactivity of CTL clone 2 was assessed using an autologous RCC cell line (1764 CTL), an autologous EBV-B cell line, an autologous fibroblast cell line, and other allogeneic RCC cell lines. RCC cells (5 × 10<sup>4</sup> cells/well), fibroblasts (5 × 10<sup>4</sup> cells), or EBV-B cells (1 × 10<sup>5</sup> cells/well) were plated in 96-well flat-bottomed plates, and 2 × 10<sup>4</sup> cells/well of CTL clone 2 were added. After a 20-h incubation, supernatants were harvested, and IFN-γ concentration was analyzed by ELISA. The HLA type of each tumor is shown on the right. Experiments were repeated at least twice, and representative data are shown.

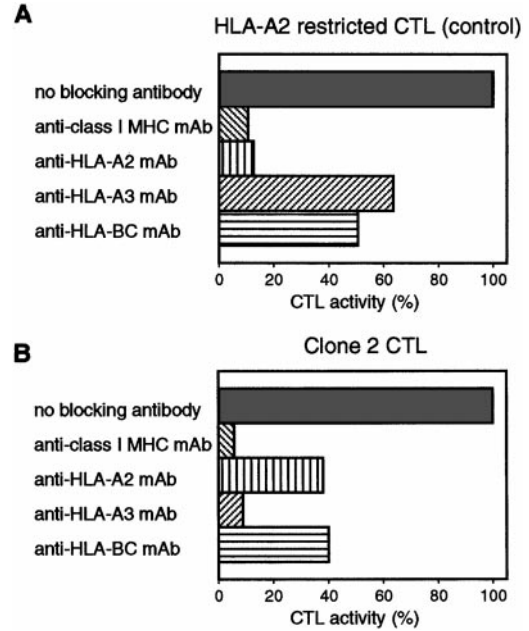


Fig. 2. Effects of various anti-HLA mAbs on the recognition of tumors by CTL clone 2. Irradiated tumor cells were incubated with mAb (20 µg/ml) for 30 min, and 2 × 10<sup>4</sup> CTLs were added. After 20 h, IFN-γ concentration in the supernatant was measured by ELISA. A, as a control, an HLA-A2-restricted RCC reactive CTL<sup>4</sup> and its autologous tumor target was used. The effect of blocking mAb on the recognition of autologous tumor by CTL clone 2 is shown in B (anti-class I MHC mAb, W6/32; anti-HLA-A2 mAb, MA2.1; anti-HLA-A3 mAb, GAPA3; anti-HLA-BC mAb, B1-23-2). Experiments were performed twice. Representative data are shown.

50°C, 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Samples were normalized by dividing by the number of copies of β-actin mRNA. The primers and the probe for FGF-5 were: forward primer, 5'-TGCAAGTGCACAGTTCACAGA-3'; reverse primer, 5'-AGTTCATGTATTGCTGAGGCATAGGTA-3'; and Taq-Man probe, 5'-6FAM-TGCAAGTTCAGGGAGCGTTTTCAAGAA-TAMRA-3'; and for β-actin were: forward primer, 5'-GGCACCAGCACAATGAAG-3'; reverse primer, 5'-GCCGATCCACACGGAGTACT-3'; and Taq-Man probe, 5'-6FAM-TCAAGATCATTGCTCCTCCTGAGCGC-TAMRA-3'. Total RNA from normal adult human organs was purchased from Clontech Laboratories (Palo Alto, CA). Total RNA from tumor cell lines was prepared by using the RNeasy mini kit (Qiagen). First-strand cDNA was synthesized by Superscript Preamplification System (Life Technologies, Inc.) using 1 µg of total RNA from either normal organs or tumor cell lines.

**Microculture of the TIL.** Fresh tumor digest was prepared as described for TIL. The digest was plated in flat-bottomed 96-well plates at the cell density of 50,000 tumor cells/well in the same media as was used for TIL culture. After 14 days, 25% of the cells in each well were transferred to 96-well flat-bottomed plates in which nonirradiated autologous RCC cells had been plated on the previous day (50,000 cells/well). On day 28, cells were washed with fresh media and used for the recognition assay. As targets, autologous-EBV-B (10,000 cells/well), autologous RCC cell line, COS-A3, and COS-A3 retrovirally transduced with full-length FGF-5 (50,000 cells/well each) were used. After 20-h incubation, IFN-γ concentration in the supernatants was measured by ELISA.

**RESULTS**

**Establishment of a CTL Clone with Specific Recognition of Autologous Renal Cell Carcinoma.** The patient is a 58-year-old Caucasian male who underwent a left radical nephrectomy in 1997. Postoperatively, several small pulmonary lesions began regressing spontaneously. In 1998, the patient was found to have a new progressive metastatic lesion in the right lung, and a second operation was

<sup>4</sup> Unpublished data.

A

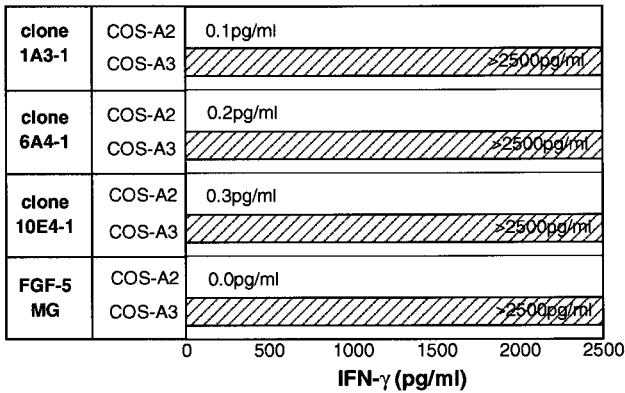
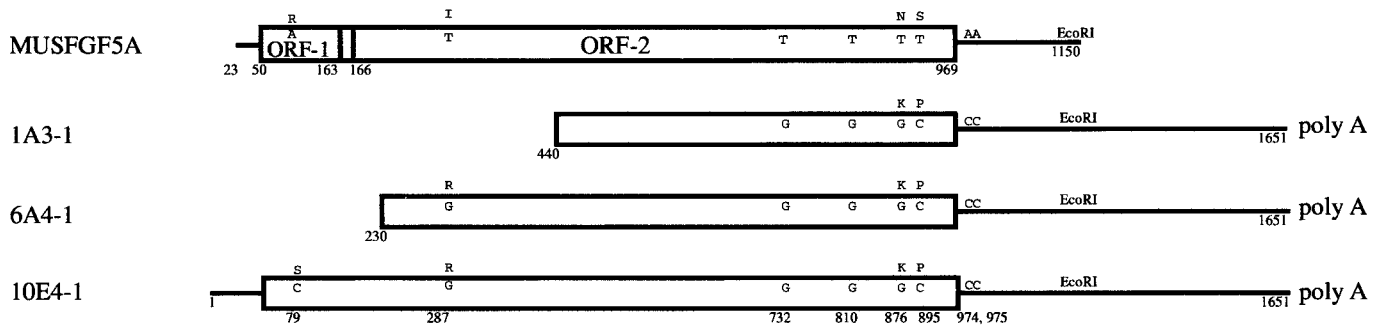


Fig. 3. A, recognition of three cDNA clones in a HLA-A3-restricted manner by CTL clone 2. Three independent clones (1A3, 6A4, and 10E4) selected after subcloning reactive cDNA pools were transfected into COS-A2 and COS-A3. The next day, CTL clone 2 ( $2 \times 10^4$  cells/well) was added, and after 20 h, supernatants were assayed for IFN- $\gamma$ . An FGF-5 gene from an independent source (FGF-5 MG) was analyzed in the same way. B, alignment of the three tumor-derived FGF-5 clones. The human FGF-5 sequence from GenBank (MUSFGF5A) is shown on the top. ORF-1 (50–166) designates a putative ORF that overlaps ORF-2 (163–969), which encodes the structural FGF-5 protein (42). Nucleotide differences from the GenBank sequence (inside each bar), their positions (under 10E4-1), and any putative amino acid changes (above each bar) are also shown.

B



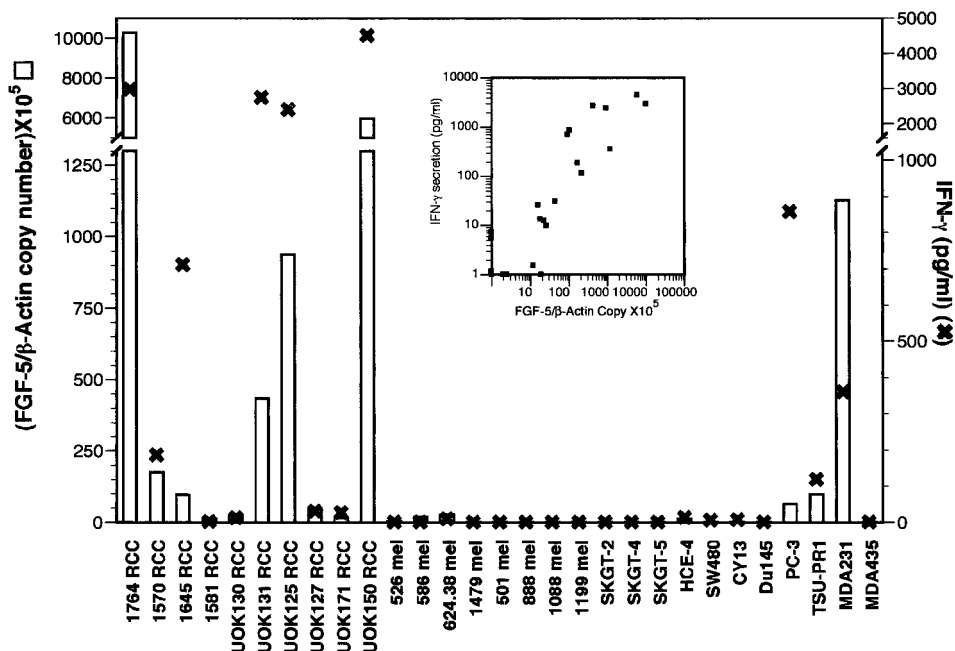
performed to resect this lesion and all of the remnants of previous lesions. One of these regressing remnants produced a TIL line when cultured in media with IL-2. This bulk TIL line was stimulated periodically with an irradiated autologous tumor cell line that had been established from the left nephrectomy specimen. After 3 months of culture, an autologous tumor-specific T-cell clone (CTL clone 2) was established by limiting dilution of the bulk TIL line. CTL clone 2 was CD3<sup>+</sup> and CD8<sup>+</sup> and was found to use V $\beta$ 12 by PCR-based T-cell receptor analysis (data not shown). The reactivity of CTL clone 2 with a panel of HLA-typed tumors (Fig. 1) suggested that CTL clone 2 recognized an antigen that was shared among renal cell carcinomas, and this recognition appeared to be restricted by HLA-A3. To confirm the restriction by HLA-A3, a blocking study using HLA-specific mAbs was performed (Fig. 2, A and B). Tumor recognition by CTL clone 2 was maximally reduced by a pan-anti-class I MHC mAb (W6/32) and an anti-HLA-A3 mAb (GAPA3), but blocking by an anti-HLA-A2 mAb (MA2.1) and an anti-HLA-B/C mAb (B1-23-2) was similar to the nonspecific effect seen with the anti-HLA-B/C antibody on an HLA-A2-restricted CTL. Collectively, it was concluded that CTL clone 2 reactivity was restricted by HLA-A3.

**Molecular Cloning of the Antigen Recognized by CTL Clone 2.** To identify the gene coding for the antigen recognized by CTL clone 2, we performed expression cloning using a plasmid-based cDNA library. To serve as the antigen-presenting target cell line, COS-7 cells were retrovirally transduced with the HLA-A3 gene derived from the autologous tumor cell line. As a control target, the HLA-A0201 gene was retrovirally transduced into COS-7 cells (hereafter referred to as COS-A3 or COS-A2, respectively). As described in “Materials and Methods,” transfectants of a cDNA library from the autologous tumor cell line were screened by assaying IFN- $\gamma$  secretion by CTL clone 2. Three independent cDNA clones that conferred recognition of COS-A3 by CTL clone 2 (Fig. 3A) were identified, and all of them were found to encode part or all of the sequence of FGF-5 (Fig. 3B).

Sequences of these clones were compared with the human FGF-5 sequence in GenBank (MUSFGF5A), and there were eight nucleotide mismatches, four of which resulted in amino acid changes. The smallest cDNA clone that was recognized by CTL clone 2 (1A3-1) had six nucleotide mismatches, and two of these changed the amino acid sequence. However, when the genomic sequence for FGF-5 was obtained from autologous EBV-B cells by direct sequencing, its sequence proved to be identical to the autologous tumor-derived sequence for FGF-5 (data not shown). In addition, FGF-5 cDNA from an independent source (FGF-5 MG; a kind gift of Dr. Mitchell Goldfarb, Mount Sinai School of Medicine, New York, NY) was also recognized by CTL clone 2 when transfected into COS-A3 (Fig. 3A). DNA sequencing of this FGF-5 MG plasmid also revealed it to be identical to our clone 1A3-1. These data corroborate that CTL clone 2 recognizes a nonmutated epitope within FGF-5.

**Expression of FGF-5 in Various Tumors and Normal Adult Tissues.** The expression of FGF-5 by tumor cell lines of various histologies and their corresponding degrees of recognition by CTL clone 2 were analyzed using q-RT-PCR and IFN- $\gamma$  release assay, respectively. Cell lines not normally expressing HLA-A3 were stably transduced with HLA-A3, and then expression was confirmed by flow cytometric analysis (data not shown). As shown in Fig. 4, 6 of 10 RCC cell lines, two of three prostate carcinoma cell lines (PC3 and TSU-PR1), and one of two breast carcinoma cell lines (MDA231) showed significant recognition by CTL clone 2 (as judged by IFN- $\gamma$  >50 pg/ml and at least twice that generated against an autologous EBV-B control). None of eight malignant melanoma cell lines (526, 586, 624.38, 1479, 501, 888, 1088, and 1199 mel), three lung carcinoma cell lines (SKGT-2, -4, and -5), one esophageal carcinoma cell line (HCE-4), or two colon carcinoma cell lines (SW480 and CY13) were recognized. As shown in the inset graph, the recognition by CTL clone 2 was highly correlated with FGF-5 copy number ( $P < 0.0001$ ). In addition, the FGF-5 nonexpressing melanoma cell lines, 888, 1088,

Fig. 4. Analysis of FGF-5 expression in various tumor cell lines and their corresponding recognition by CTL clone 2 (RCC; mel, malignant melanoma; SKGT2, 4, and 5, lung carcinoma; HCE-4, esophageal carcinoma; SW480 and CY13, colon carcinoma; Du145, PC-3, and TSU-PR1, prostate carcinoma; and MDA231 and MDA435, breast carcinoma). FGF-5 expression was analyzed by q-RT-PCR. FGF-5 copy number was normalized to  $\beta$ -actin copy number in each cell line, and FGF-5 copy number/ $10^5 \beta$ -actin copy number was plotted in flat-bottomed 96-well plates, and  $2 \times 10^4$  CTL clone 2 were added. After 20 h, supernatants were assayed for IFN- $\gamma$  (shown by X). In the absence of HLA-A3 transfection, there was no recognition of any HLA-A3-negative line (data not shown). In the insert graph, the correlation between FGF-5 expression and recognition by the CTL ( $P < 0.0001$ ) is shown.



1199, and 1479 mel, were recognized by the CTL when transduced with the *FGF-5* gene, further corroborating the correlation between FGF-5 expression and the recognition by CTL clone 2 (data not shown). In normal tissues, FGF-5 was only detectable in brain and kidney (data not shown). However, the expression level of FGF-5 in these tissues ( $\sim 20$  FGF-5 copies/ $10^5 \beta$ -actin copies) would be predicted to result in IFN- $\gamma$  secretion of  $\leq 20$  pg/ml, compared with 2980 pg/ml released in response to the autologous tumor cell line (Fig. 4).

**Rediscovery of Anti-FGF-5 Activity in an Independent Metastatic Lesion.** Because clone 2 CTL was established by repeated *in vitro* stimulation and limiting dilution, it was possible that it represented a rare reactivity that was amplified *in vitro*. To address this possibility, we looked for anti-FGF-5 activity in an independent lung metastatic lesion using a minimum of *in vitro* stimulation. Micro-TIL culture in 96-well plates was initiated using a fresh tumor digest of an independent lung metastatic lesion from the same patient. The cultures were stimulated once on day 14 with the autologous tumor cell line and were analyzed for anti-FGF-5 reactivity on day 28. Most of the wells (457 of 480 wells) were dominated by nonspecific activities that reacted with both autologous tumor cell line and EBV-B cells. However, 23 wells showed specific recognition of the autologous tumor (representative wells shown in Fig. 5, microcultures 1 to 3), and four of these recognized FGF-5 (Fig. 5, microcultures 4 to 7). In another experiment, CD8<sup>+</sup> cells were purified from the fresh tumor digest using antibody-conjugated magnetic beads and were directly subjected to limiting dilution. In this experiment, 1 clone of 288 clones/clonoids was found to recognize FGF-5 (data not shown). These findings indicate that CTL precursors reactive with FGF-5 were present at significant frequencies in multiple fresh tumor preparations in this patient.

**DISCUSSION**

In the present report, we describe the establishment of a CD8<sup>+</sup> CTL clone from a clinically regressing renal cancer metastasis that demonstrates specific recognition of the autologous RCC cell line. In our experience, as well as that of other investigators, the reactivities of RCC-TIL have been nonspecific (18), and limiting dilution cloning seems to be an important step for the identification of autologous

RCC-specific CTL activity. CTL clone 2 recognizes multiple RCC cell lines in an HLA-A3-restricted manner, and the antigen was identified to be FGF-5 by expression cloning. This FGF-5 clone has no tumor-specific mutations as demonstrated by determining the FGF-5 genomic sequence from autologous EBV-B cells and by recognition of COS-A3 when transfected with an *FGF-5* gene from an independent source. By q-RT-PCR, FGF-5 was shown to be overexpressed in about 60% of RCC cell lines, two of three prostate carcinoma cell lines, and one of two breast carcinoma cell lines. The recognition of these cell lines by CTL clone 2 coincided well with the degree of overexpression of FGF-5.

The HLA-A3-restricted epitope in FGF-5 has not yet been identified. Truncations of the *FGF-5* gene have shown that CTL recognition can be conferred by transfecting the coding sequence for as few as 60 amino acids from FGF-5 (full-length FGF-5 is comprised of 268 amino acids). However, further truncation from either 5' or 3' of the gene resulted in the loss of recognition by the CTL (data not shown). In addition, 9-, 10-, 12-, and 16-mer peptides synthesized from that

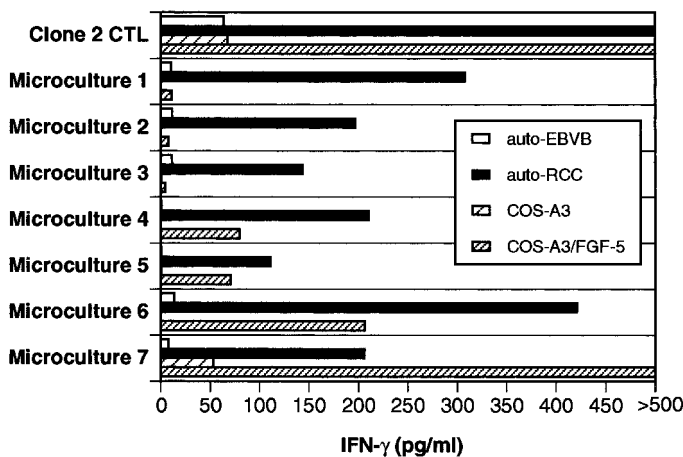


Fig. 5. Reidentification of the anti-FGF-5 reactivity in the short-term culture. Microculture was set up as described in the "Materials and Methods." Reactivity against autologous EBV-B, autologous RCC cell line, COS-A3, and COS-A3 stably transfected with *FGF-5* gene was analyzed by IFN- $\gamma$  ELISA.

region could not reconstitute the CTL recognition. At this point, posttranslational modification such as glycosylation (19–20), cysteinylolation (21), or phosphorylation (22) is hypothesized to be playing a role in the generation of the epitope. Attempts to identify the epitope by mass-spectrometry analysis (23) are under way.

Ideally, tumor antigens for cancer therapy should satisfy several requirements. Firstly, they should be expressed at recognizable levels in tumors and not in normal tissues. According to expression analysis in mice, FGF-5 is expressed in a complex spatiotemporal pattern during embryogenesis. However, in adults, expression in normal tissues has been consistently observed only in the brain and at low levels (24). In this study, normal adult brain and kidney were found to express FGF-5 by q-RT-PCR analysis. Comparison of FGF-5 expression in various tumor cell lines and their recognition by the CTL clone 2 enabled us to estimate the threshold of FGF-5 expression necessary for the recognition by clone 2. This threshold for recognition was approximately 0.1% of the  $\beta$ -actin mRNA copy number, and brain and kidney expression of FGF-5 was well below this level. In addition, 4 of 10 RCCs showed more than 1000 times higher expression of FGF-5 compared with these normal tissues, and this differential expression supports a high therapeutic ratio between antitumor effects and the induction of autoimmunity. In addition, some central nervous system sites appear privileged from autoimmune attack, as evidenced by the lack of retinal toxicity with either vaccination or cell transfer targeting antigens shared by melanomas and retinal pigment epithelium. Secondly, an ideal antigen is one that is widely expressed on tumors. Although tumor-specific mutations may be potent antigens, unless they are common mutations, they have limited practical use in defined-antigen vaccines. Nonmutated, tumor-expressed (or overexpressed) antigens have been more readily integrated into vaccine trials, particularly in the treatment of patients with melanoma. FGF-5 is a nonmutated antigen, overexpressed in some RCCs, breast carcinomas, and prostate cancers. According to the literature, it is also reported to be overexpressed in bladder cancers and pancreatic cancers (25, 26). Therefore, it represents an attractive tumor antigen for a number of cancers that lack identified vaccine targets. Thirdly, it is desirable that a tumor antigen have an important biological function in maintaining the malignant phenotype. If this is not the case, tumor heterogeneity can result in the appearance of antigen-negative tumor clones that can evade immune attack. The pigment-related antigens in melanoma may be susceptible to such evasion because they are not important in maintaining the malignant phenotype (27). FGF-5 was originally identified as an oncogene that transformed NIH-3T3 cells (28). In pancreatic cancer, autocrine and paracrine growth-promoting activities of FGF-5 have been proposed (26). Another potentially important activity of FGF-5 is its function as an angiogenic factor (24, 29). It has activity in myocardial revascularization models, and the role of tumor-produced angiogenic factors has been of significant clinical and experimental interest for many malignancies including RCC (30).

Most tumor-associated antigens have been categorized in three groups: (a) tissue differentiation antigens [e.g., MART-1 (31), tyrosinase (32), and gp100 (33)]; (b) tumor-specific mutations [e.g.,  $\beta$ -catenin (34) and CASP-8 (35)]; and (c) proteins sharing expression on tumor and testis [e.g., MAGE-1 (36), BAGE (37), and NY-ESO-1 (38)]. Recently, antigens overexpressed on tumors but not expressed by testis have been described (e.g., the telomerase catalytic subunit; Ref. 39). FGF-5 appears to belong to this new class of tumor antigens. The antigens that belong to this group can be identified not only by the CTL-based expression cloning approach but also by investigating the immune response to proteins that are known to be overexpressed in tumors (40, 41). As more such antigens are identified, they may

represent attractive targets for vaccination strategies and allow such strategies to be applied to many more patients with cancer.

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