Abrogation of fibroblast activation protein enzymatic activity attenuates tumor growth

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Abstract

Tumor-associated fibroblasts are functionally and phenotypically distinct from normal fibroblasts that are not in the tumor microenvironment. Fibroblast activation protein is a 95 kDa cell surface glycoprotein expressed by tumor stromal fibroblasts, and has been shown to have dipeptidyl peptidase and collagenase activity. Site-directed mutagenesis at the catalytic site of fibroblast activation protein, Ser624Ala, resulted in an ~100,000-fold loss of fibroblast activation protein dipeptidyl peptidase (DPP) activity. HEK293 cells transfected with wild-type fibroblast activation protein, enzymatic mutant (S624A) fibroblast activation protein, or vector alone, were inoculated subcutaneously into immunodeficient mouse to assess the contribution of fibroblast activation protein enzymatic activity to tumor growth. Overexpression of wild-type fibroblast activation protein showed growth potentiation and enhanced tumorigenicity compared with both fibroblast activation protein S624A and vector-transfected HEK293 xenografts. HEK293 cells transfected with fibroblast activation protein S624A showed tumor growth rates and tumorigenicity potential similar to vector-transfected HEK293. In vivo assessment of fibroblast activation protein DPP activity of these tumors showed enhanced enzymatic activity of wild-type fibroblast activation protein, with only baseline levels of fibroblast activation protein DPP activity in either fibroblast activation protein S624A or vector-only xenografts. These results indicate that the enzymatic activity of fibroblast activation protein is necessary for fibroblast activation protein–driven tumor growth in the HEK293 xenograft model system. This establishes the proof-of-principle that the enzymatic activity of fibroblast activation protein plays an important role in the promotion of tumor growth, and provides an attractive target for therapeutics designed to alter fibroblast activation protein–induced tumor growth by targeting its enzymatic activity. [Mol Cancer Ther 2005;4(3):351–60]

Introduction

Tumor-associated fibroblasts are functionally and phenotypically distinct from normal fibroblasts that are not in the tumor microenvironment. Tumor fibroblasts synthesize a wide variety of factors that can potentiate tumor growth and invasion. Fibroblasts normally provide mechanical support for tissue through the synthesis of extracellular matrix proteins. Fibroblasts in the tumor stroma have also been shown to express and secrete a large variety of proteases including stromelysin-3, plasminogen activator, and collagenase IV (1–3). Although some malignant cells can also produce proteases, the majority of protease production derives from the host stroma rather than from the tumor cells themselves (4). In addition to proteases, tumor stromal fibroblasts also play a critical role in the tumor microenvironment by providing growth factors and adhesion molecules that contribute to tumor invasion and motility (5–8). The mechanistic pathways by which tumor stromal fibroblasts contribute to the invasive and metastatic behavior of malignant cells are starting to be understood. The selectivity of fibroblast activation protein expression in the tumor stroma suggests that it may play a significant role in stromal contributions to tumor growth and invasion.

Fibroblast activation protein is a type II integral membrane glycoprotein belonging to the serine protease family. Fibroblast activation protein is a 95 kDa cell surface glycoprotein expressed by tumor stromal fibroblasts. Fibroblast activation protein has been shown to have dipeptidyl peptidase (DPP) and collagenase activity (9, 10). Fibroblast activation protein is well expressed by reactive stromal fibroblasts in >90% of human epithelial carcinomas (breast, lung, colorectal, and ovary) as determined by immunohistochemistry (11). Neural and lymphoid cells, as well as surrounding normal tissue, do not express fibroblast activation protein. Fibroblast activation protein expression in normal tissue has only been seen in a subset of pancreatic endocrine cells, and transiently in healing wounds. Consistent with its expression in areas of wound-healing fibrosis, fibroblast activation protein has been seen in the tissue-remodeling interface in areas of chronic inflammation, such as liver cirrhosis (12). Epithelial...
Fibroblast Activation Protein Inhibition Attenuates Tumors

Materials and Methods

Modulated Protein Structure of Murine Fibroblast Activation Protein

The murine fibroblast activation protein extracellular domain was modeled from the crystal structure of DPP IV (DPPIV) in the Protein Data Bank (22). PSI-BLAST (23) was used to search the nonredundant protein sequence database to construct a position-specific similarity matrix for the sequence of fibroblast activation protein. We searched a sequence database of proteins in the Protein Data Bank using the position-specific matrix, and chose to use the human DPPIV Data Bank entries 1N1M and 1NU8 (to model the inhibitor) as templates, the alignment having an expectation value of 0.0 and a sequence identity of 53% over 722 amino acids of fibroblast activation protein (residues 38-759). This covered all eight blades of the propeller and the hydrolase domain. Side chain conformations for fibroblast activation protein were built onto the backbone structure of DPPIV with the program SCWRL (24), keeping conserved amino acids fixed in their crystallographic Cartesian coordinates. Loopy (25) was used for loop modeling. The software package Chimera5 (26) was used for molecular graphics.

HEK293 Xenografts

Murine fibroblast activation protein cDNA was obtained from primary mouse embryonic fibroblast cultures as previously described (21). Fibroblast activation protein wild-type DNA was enzymatically mutated by site-directed mutagenesis of Ser624 to Ala624. Mutagenic primer 5'-GCC-ATATGGGGCTGGGCCCTACGGAGG-3' were designed to flank the fibroblast activation protein DNA template and alter serine (TCC) at 624 to alanine (GCC). PCR amplification of the mutant DNA was engineered by annealing the mutagenic primers to parental fibroblast activation protein DNA template using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Dpr1 restriction enzyme was added for digestion of parental, nonmutated DNA. The mutant clone S624A was transformed into XL10-Gold ultracompetent cells by heat pulsation at 42°C for 30 seconds. The enzymatically mutated fibroblast activation protein was cloned into pSec/Hygro B plasmid, and expressed by HEK293 cells under antibiotic selection pressure. C.B17/ICr-scid mice were injected s.c. with 7 x 10^6 HEK293 cells transfected with fibroblast activation protein wild-type, fibroblast activation protein S624A, or vector alone. Animals were maintained under pathogen-free conditions in autoclaved microisolator cages in the Fox Chase Cancer Center Laboratory Animal Facility. Serial tumor measurements were obtained every 3 to 4 days by caliper in three dimensions. Tumor volumes were calculated by the formula volume = height x weight x length x 0.5236. Animals were followed until any mouse developed a tumor measuring > 2 x 2 x 2 cm, was observed to be suffering, or seemed moribund. Animals were euthanized according to institutional policy.

In vivo Assay of Fibroblast Activation Protein Dipeptidyl Peptidase Activity

This assay was done as described previously (21), with the following modifications. To determine the degree of fibroblast activation protein enzymatic activity in tumors, an immunocapture assay was done with Ala-Pro-7-amido-4-trifluoromethylcoumarin (Ala-Pro-AFC) as a substrate. Ninety-six-well Fluoronunc MaxiSorb plates were coated overnight at 4°C with anti-fibroblast activation protein rabbit polyclonal antibody obtained as previously described (21) at a 100 µg/mL dilution. Plates were rinsed with wash buffer consisting of PBS and 0.1% Tween 20, and

5 http://www.cgl.ucsf.edu/chimera

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blocked with 5% bovine serum albumin for 1 hour at room temperature. HEK293 or HT-29 xenografts were excised and proteins extracted using the detergent tissue protein extraction reagent (Pierce, Rockford, IL) according to the manufacturer’s instructions. Tumors were homogenized for 60 seconds in the tissue protein extraction reagent at a concentration of 10 mL/g tumor, and samples centrifuged at 14,000 rpm × 10 minutes to pellet the cellular debris. Protein concentrations were assessed and normalized by spectrophotometric determination and bicinchoninic acid assay (Pierce). One milligram of the total tumor protein extracts, which contained fibroblast activation protein, was added to the wells and incubated for 1 hour, washed 10 times with PBS 0.1% Tween 20, and DPP activity assessed by cleavage of 0.25 mmol/L Ala-Pro-AFC for 1 hour at room temperature. Release of the free AFC fluorescent substrate was detected on a cytofluor fluorimeter (Labsystems, Helsinki, Finland) with 396 nm excitation and 490 emission.

**Fibroblast Activation Protein Activity Assay**

$K_m$ and $V_{max}$ values for fibroblast activation protein were estimated from data that measured the release of free AFC from the Ala-Pro-AFC substrate after incubation with fibroblast activation protein. The extracellular domain of both wild-type fibroblast activation protein and S624A fibroblast activation protein were recombinantly expressed, as previously described (21). Assays containing concentrations of 0.1, 0.25, 0.5, 1, and 5 mmol/L of the Ala-Pro-AFC substrate were done in triplicate using serial concentrations of fibroblast activation protein (100, 200, 1,000, 2,000, and 5,000 ng). Assays were done in 100 mmol/L Tris, 100 mmol/L NaCl (pH 7.8) at room temperature with reaction times varying from 5 minutes to 1 hour for wild-type fibroblast activation protein, and required extension of up to 24 hours in order to see product formation with the fibroblast activation protein S624A. Selecting only that data which estimated the initial rate and was within the linear region of the spectrofluorimeter, $K_m$ and $V_{max}$ values were determined by fitting a hyperbolic equation to the rate versus substrate concentration data using the program SigmaPlot (Statistical Package for the Social Sciences, Chicago, IL).

**Immunohistochemistry**

As previously described (21), HEK-fibroblast activation protein tumors from C.B17/Icr-SigmaPlot (Statistical Package for the Social Sciences, Rockford, IL) biotin-streptavidin detection system (San Ramon, CA) was used with MOPC-21 was used as an irrelevant control antibody. The entire procedure was done on ice.

**Immunofluorescence**

HEK293 cells (1 × 10^6) transfected with either wild-type fibroblast activation protein or S624A fibroblast activation protein were cultured on a cover slip overnight. HEK293-transfected cells were washed in PBS, then fixed using PBS containing 5% sucrose and 4% paraformaldehyde for 20 minutes. After rehydration in PBS, the cells were blocked for 1 hour with 10% bovine serum albumin and then washed in PBS. The cells were subsequently incubated for 60 minutes with 10 µg/mL of the primary rabbit monoclonal antibody 6E1, washed thrice, and incubated for 30 minutes with 3 µg/mL of the secondary rhodamine red donkey anti-rabbit F(ab')2 (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS with 3% bovine serum albumin. The cells were washed thrice for 10 minutes in PBS, and once in distilled water. Cover slips were mounted with Vectashield (Vector Lab, Burlingame, CA) with 4',6-diamidino-2-phenylindole for nuclear staining.

**Western Analyses**

SDS-PAGE gels (10%) were loaded with 200 µg of tissue protein extraction reagent extracted proteins from HT-29 xenografts and transferred to a nitrocellulose membrane. After blocking with 10% milk in PBS + 0.1% Tween, the rabbit polyclonal antibody targeting murine fibroblast activation protein at a dilution of 20 µg/mL, and the secondary antibody of donkey anti-rabbit horseradish peroxidase (Amersham, Piscataway, NJ) at a 1:1,000 dilution (v/v) was used. Films were developed using Supersignal West Pico Chemiluminescent Substrate (Pierce). Anti-β-actin antibody (Sigma, St. Louis, MO) in a 1:1,000 dilution was used as a control of loaded protein.

**In vitro and In vivo Assay of Val-boroPro**

Val-boroPro is a boronic acid inhibitor of fibroblast activation protein enzymatic activity. To determine the pH dependence of Val-boroPro in inhibiting fibroblast activation protein enzymatic activity, Val-boroPro was incubated overnight either in 0.01 N HCl or neutral reaction buffer consisting of 100 mmol/L Tris, 100 mmol/L NaCl (pH 7.8). Serial 10-fold dilutions of Val-boroPro were incubated with 3 mmol/L recombinant murine fibroblast activation protein-extracellular domain at room temperature for 15 minutes prior to the addition of 0.25 mmol/L Ala-Pro-AFC substrate to give final inhibitor concentrations ranging from 4 × 10^{-4} to 4 × 10^{-12} mol/L. Inhibition of enzymatic activity was analyzed using a cytofluor fluorimeter and normalized as the percentage of baseline release of free AFC by fibroblast activation protein without inhibitor.
The *in vivo* effects of Val-boroPro on tumor growth were assessed in cohorts of five C.B17/1cr-scid mice inoculated with 5 × 10^6 HT-29 colorectal cancer cells s.c. in the flank. Mice were treated with either 50 μg of the fibroblast activation protein inhibitor Val-boroPro in 100 μL normal saline, or 100 μL normal saline by gastric gavage daily for 3 weeks (days 1-21 of inoculation). Animals were maintained under pathogen-free conditions in autoclaved microisolator cages in the Fox Chase Cancer Center Laboratory Animal Facility. Serial tumor measurements were obtained twice a week by caliper in three dimensions. Tumor volumes were calculated by the formula volume = height × weight × length × 0.5236. Animals were followed until any mouse developed a tumor measuring > 2 cm, was observed to be suffering, or seemed moribund. Animals were euthanized according to institutional policy.

**Statistical Method**

A random coefficient model was applied to model the change of tumor size over time and examine the treatment effect on tumor growth. The tumor size for each animal was modeled as a quadratic function over time. Welch’s variance-weighted one-way ANOVA with correction for unequal variances was used to compare treatment groups with respect to tumor size at each time point. Repeated measure ANOVA approach was used to compare fluorescence measures of fibroblast activation protein enzymatic activity among treatment groups. All the statistical analyses were carried out using SAS software and *P* values less than 0.05 indicated statistical significance.

**Results**

**Revised Model of Fibroblast Activation Protein**

We have previously described the modeled structure of murine fibroblast activation protein based on the crystal structure of prolyl oligopeptidase, which shares a 12% sequence identity with fibroblast activation protein (21). The recent availability of the crystal structure of DPPIV (22), which shares a 53% sequence homology with fibroblast activation protein allowed the construction of a revised model of murine fibroblast activation protein as shown in Fig. 1. The modeled structure consists of a short cytoplasmic tail (amino acids 1-6), a single hydrophobic transmembrane region (amino acids 7-25), and a large extracellular domain (amino acids 26-761) consisting of two major domains: (a) bladed β-propeller domain (amino acids 99-499), and (b) αβ-hydrolase domain (amino acids 500-761) that contains the catalytic triad. The catalytic triad consists of a serine at position 624, aspartic acid at 702, and histidine at 734. The availability of the DPPIV crystal structure allowed a complete modeling of the eight-blade β-propeller component that was just predicted in the previous model.
Although there is a four-residue difference between DPPIV and fibroblast activation protein, the model also predicts that fibroblast activation protein forms homodimers with a larger side hole of ~25 Å, and a second smaller opening in the β-propeller domain. The model predicts that site-directed mutation of Ser624 will induce loss of fibroblast activation protein DPP activity. The revised model also predicts that fibroblast activation protein forms homodimers with a larger side hole of ~25 Å, and a second smaller opening in the β-propeller domain of ~10 Å. Although there is a four-residue difference between DPPIV and fibroblast activation protein out of 28 side chains in the dimer interface (T256 → S279, T246 → R140, G227 → Q231, and D275 → N215), this resulted in replacement of a hydrogen bond (T256-D275 → R216-Q211), and is unlikely to influence the homodimerization interface stability of the modeled fibroblast activation protein.

**Fibroblast Activation Protein Growth Potentiation Is Dependent on Its Enzymatic Activity**

Serial substrate dilutions of Ala-Pro-AFC were incubated with recombinantly expressed fibroblast activation protein-extracellular domain, and the data fitted to the Michaelis-Menten equation yielding a wild-type fibroblast activation protein $K_m$ of ~0.25 mmol/L, with a $V_{max}$ of 55 mmol/L/minute/µg. In contrast, site-directed mutagenesis of the catalytic site of fibroblast activation protein S624A resulted in an enzymatic mutant with ~10,000- to 100,000-fold loss of fibroblast activation protein DPP activity at 1 mmol/L substrate concentration. Full-length fibroblast activation protein S624A was expressed by HEK293 cells. This enzymatically inactive mutant was utilized to assess the contribution of fibroblast activation protein enzymatic activity to tumor growth as compared with our previously described construct of wild-type fibroblast activation protein (21). Immunofluorescence and flow cytometry under nonpermeabilized conditions confirmed the cell surface presence of both wild-type and S624A fibroblast activation protein transfections of HEK293 cells (data not shown).

C.B17/1cr-scid mice were injected s.c. with HEK293 cells that had been transfected with wild-type, and S624A fibroblast activation proteins, or vector alone (mock), and serial tumor measurements were obtained. The growth potentiation of wild-type fibroblast activation protein compared with vector-transfected HEK293 xenografts was confirmed, as shown in the tumor growth curves for all 18 mice in each cohort (Fig. 2). Enhanced tumorigenicity of fibroblast activation protein overexpressing tumors was also seen, as all 18 mice inoculated with $7 \times 10^6$ HEK293-fibroblast activation protein wild-type developed tumors by day 49, when mice with large tumors were euthanized according to institutional policy. In contrast, only 13 of 18 and 11 of 18 mice inoculated with HEK293-fibroblast activation protein S624A or HEK293-vector only cells developed tumors at day 49, respectively ($P = 0.046$ comparing HEK-fibroblast activation protein versus HEK-fibroblast activation protein S624A). In addition, HEK293-fibroblast activation protein S624A xenografts showed tumor growth rates similar only to vector-transfected HEK293 controls (Fig. 2). These results indicate that the enzymatic activity of fibroblast activation protein is necessary for fibroblast activation protein-driven tumor growth in fibroblast activation protein transfected HEK293 cells.

**In vivo Determination of Fibroblast Activation Protein in Total Protein Lysates from Fibroblast Activation Protein Transfected HEK293 Cells**

In vivo determination of fibroblast activation protein DPP enzymatic activity in the tumor xenografts was assessed. Rabbit polyclonal antibodies were used to capture fibroblast activation protein protein within total protein lysates from fibroblast activation protein transfected HEK293 xenografts obtained 21 days after inoculation into the flanks of C.B17/1cr-scid mice. Significantly...
enhanced DPP activity was seen in wild-type fibroblast activation protein compared with fibroblast activation protein S624A or vector only controls as seen in Fig. 2 (bottom). Thus, fibroblast activation protein–mediated growth potentiation of HEK293 tumor xenografts is associated with enhanced DPP enzymatic activity within tumors. These results extend our previous observations and support the premise that specific inhibition of fibroblast activation protein enzymatic activity may provide a therapeutic intervention by which tumor growth is attenuated.

**Immunohistochemical Analyses of Xenografts**

Immunohistochemistry studies done on the HEK293 xenografts showed cell surface membrane staining of the individual HEK293 cells transfected with wild-type fibroblast activation protein as seen in Fig. 3. Interestingly, immunohistochemical analysis of tumors obtained from mice inoculated with HEK293-fibroblast activation protein S624A showed infrequent fibroblast activation protein expression by the individual HEK293 cells. Furthermore, when fibroblast activation protein expression was seen in the HEK293-fibroblast activation protein S624A cells, it was cytoplasmic rather than on the cell membrane. This was surprising given the robust protein presence of fibroblast activation protein S624A on the surface of the inoculated HEK293 cells that was readily detected by both flow cytometry and immunofluorescence. Although HEK293-fibroblast activation protein S624A cells in culture can exhibit the presence of both cell surface and cytoplasmic fibroblast activation protein S624A (data not shown), the expression levels of fibroblast activation protein at the cell surface of HEK293 fibroblast activation protein wild-type and HEK293 fibroblast activation protein S624A cells are similar. However, in vivo inoculation of HEK fibroblast activation protein S624A cells results in greatly diminished fibroblast activation protein S624A expression and loss of cell surface localization (Fig. 3C and D). This suggests that the enzymatically inactive fibroblast activation protein not only fails to provide the growth advantage of wild-type fibroblast activation protein (as seen in Fig. 2), but that in vivo negative selection occurs with respect to both expression and localization of the enzymatically inactive protein.

**Inhibition of Fibroblast Activation Protein Enzymatic Activity Is Associated with Growth Attenuation of HT-29 Xenografts That Induce Stromal Expression of Fibroblast Activation Protein**

Recombinant murine fibroblast activation protein–extracellular domain was incubated with Val-boroPro prior to the addition of Ala-Pro-AFC as a fluorescent substrate. This yielded in vitro inhibition of fibroblast activation protein enzymatic activity with an IC$_{50}$ of $4 \times 10^{-8}$ mol/L.
(pH 2) as shown in Fig. 4. This inhibition is pH-dependent, with a significant loss of inhibitory potency at neutral pH, as Val-boroPro remains in linear form at acidic pH, but cyclizes at neutral pH.

Xenografts of the HT-29 colorectal cancer cell line in C.B17/Icr-scid mice induce expression of fibroblast activation protein on host tumor stromal fibroblasts. The HT-29 cell line itself does not express fibroblast activation protein (data not shown), but induces the expression of fibroblast activation protein in the surrounding tumor stroma of HT-29 xenografts as we have previously shown (21). Western analysis using rabbit polyclonal antibodies confirms the expression of murine fibroblast activation protein in the HT-29 xenograft protein lysates. Figure 5 shows the in vivo tumor growth attenuation of HT-29 xenografts treated by gastric gavage with the fibroblast activation protein inhibitor Val-boroPro once daily for 21 consecutive days. On day 24 after inoculation, Val-boroPro-treated animals had a 45% reduction in HT-29 xenograft tumor size compared with normal saline–treated controls (459.0 ± 107.2 mm³ versus 837.0 ± 39.7 mm³, respectively). No obvious toxicities were observed in any of the animals treated with this boronic acid small molecule inhibitor of DPP activity. The degree of in vivo inhibition of fibroblast activation protein DPP activity at the tumor site after treatment with Val-boroPro was also assessed. Tumor protein extracts of HT-29 xenografts from mice treated for 7 days with 50 μg/day of Val-boroPro by gastric gavage were assessed for fibroblast activation protein enzymatic activity. Using an immunocapture assay of fibroblast activation protein from these tumors, a 44% inhibition of fibroblast activation protein enzymatic activity was seen in tumors treated with Val-boroPro compared with normal saline controls (105.5 ± 33.0 versus 194.2 ± 25.9 A.U., respectively; P = 0.046). Representative samples from three mice treated with Val-boroPro show similar expression levels of fibroblast activation protein expression in both treated and untreated tumors. A protein loading control as assessed by anti-actin antibody was used.

Discussion
The studies reported here show the proof-of-principle that inhibition of the DPP activity of fibroblast activation protein, a stromal selective protein, attenuates the growth of tumors treated by Val-boroPro. This was accomplished using a tumor model that recapitulates human epithelial cancer biology, with induction of fibroblast activation protein expression at tumor sites, but not fibroblast activation protein expression levels following treatment by Val-boroPro. This is in vivo attenuation of fibroblast activation protein enzymatic activity in vitro, and attenuates tumor growth in vivo. In addition, pharmacodynamic assessment of fibroblast activation protein DPP activity in vitro of the HT-29 xenografts shows a reduction of fibroblast activation protein enzymatic activity at tumor sites, but not fibroblast activation protein expression levels following treatment by Val-boroPro. This was accomplished using a tumor model that recapitulates human epithelial cancer biology, with induction of fibroblast activation protein in the tumor stroma.

Figure 4. In vitro inhibition of fibroblast activation protein by Val-boroPro. The pH dependence of Val-boroPro in inhibiting fibroblast activation protein enzymatic activity is shown using serial dilutions of Val-boroPro in either 0.01 N HCl (pH 2) or neutral reaction buffer (pH 8). Inhibition of fibroblast activation protein DPP enzymatic activity by Val-boroPro was analyzed using a cytofluor fluorimeter and normalized as the percentage of baseline release (without inhibitor) of free AFC by fibroblast activation protein cleavage of the Ala-Pro-AFC substrate. A two-log greater potency of Val-boroPro in acidic pH was seen with a Kd of 4.35 × 10⁻⁸ and 4.16 × 10⁻⁷ mol/L for pH 2 and pH 8, respectively.
of epithelial carcinomas. This extends our previous observation that overexpression of fibroblast activation protein wild-type protein confers a tumor growth advantage by demonstrating that the enzymatic activity of fibroblast activation protein is responsible for this growth advantage. Transfected HEK293 cells were chosen for study in an animal model due to its modest tumor growth rates, and the ability of these cells to readily express transfected mammalian proteins. Although HEK293 cells are epithelial and not stromal in origin, overexpression of fibroblast activation protein in this model system provides the proof-of-principle of the role of fibroblast activation protein in tumor growth and invasion, and may be akin to clinical cancers characterized by overexpression of fibroblast activation protein in the tumor cell itself as seen in certain sarcomas (13). We also provide evidence of the role of fibroblast activation protein enzymatic activity in a stromal selective model of fibroblast activation protein using HT-29 colorectal cancer xenografts.

The mechanistic pathway(s) by which fibroblast activation protein potentiates tumor growth is not known, as natural substrates of fibroblast activation protein have not yet been identified. Given the dual enzymatic activity of fibroblast activation protein as both a DPP and a collagenase, multiple substrates with distinct biological effects may be found. It is tempting to speculate that fibroblast activation protein may degrade extracellular matrix via its collagenase activity, while regulating other hormones through its DPP cleavage of a penultimate alanine or proline. Intriguingly, seprase forms complexes with α3β1 integrin in the presence of collagen (27), suggesting that fibroblast activation protein may promote an invasive phenotype through cell adhesion pathways. The dual specificity of fibroblast activation protein for both DPP and collagenase activity utilizes the same serine catalytic site (10), and thus can be targeted for therapeutic effect. Using an enzymatically inactive fibroblast activation protein mutant, we provide the first direct evidence that abrogation of fibroblast activation protein enzymatic activity in vivo results in tumor growth attenuation of fibroblast activation protein–driven growth.

We find that fibroblast activation protein overexpression potentiates tumor growth that is dependent on this protein’s enzymatic activity. This contrasts with a recent report suggesting that fibroblast activation protein may be a tumor suppressor, through mechanisms that are independent of its enzymatic activity, Ramirez-Montagut et al. (28) reported that fibroblast activation protein reexpression by malignant melanoma cells abrogated tumorigenicity, and that this effect was independent of its enzymatic activity. In fact, the enzymatic mutant was a more potent tumor suppressor than wild-type fibroblast activation protein in this melanoma system. One possible explanation for such a disparate set of conclusions is that HEK293 cells are epithelial, whereas melanoma cells have mesenchymal features. Although melanocytes lose fibroblast activation protein expression with malignant transformation, expression of stromal fibroblast activation protein enzymatic activity is enhanced in the process of melanoma carcinogenesis (29). We speculate that a negative biological consequence for melanoma cells that express fibroblast activation protein leads to an induction or outsourcing of fibroblast activation protein functions to the surrounding stromal fibroblasts. Thus, normal physiologic processes of fibroblast activation protein that are seen in tadpole tail resorption and wound healing (11, 30, 31) may be usurped in the activated tumor stroma to promote an invasive tumor phenotype. This growth potentiation effect of fibroblast activation protein/seprase was recently independently confirmed in breast cancer (32).

Although the vast majority of epithelial malignancies induce expression of human fibroblast activation protein by tumor stromal fibroblasts, a small proportion of clinical epithelial tumors invade and metastasize independently of fibroblast activation protein. Clearly, factors other than fibroblast activation protein contribute to tumor invasion. Tumor growth independent of fibroblast activation protein expression is seen in fibroblast activation protein–negative tumors, both in the clinic and in the studies reported here. Other stromal factors that participate in tumor growth, or “the company fibroblast activation protein keeps,” are the subject of ongoing investigations. We also cannot exclude the possibility that fibroblast activation protein may interact with other proteins that regulate tumor growth independent of its enzymatic activity. Tumor growth rates did not differ between the HEK293 xenografts transfected with either fibroblast activation protein S624A or the empty vector, although there was a slight trend favoring HEK293-fibroblast activation protein S624A compared with HEK293-vector xenografts. Despite these caveats, fibroblast activation protein enzymatic function provides a potentially important new therapeutic target that could be applicable to the therapy of a variety of human malignancies.

Dipeptides containing the α-amino boronic acid analogue of proline, boroPro, are potent small molecule inhibitors of DPP enzymatic activity (33, 34). These boronic acid compounds are reversible transition state analogues, demonstrating Ki values in the picomolar range (35), and have known in vivo biological effects (36), including antitumor effects (37). The studies reported here show the ability of Val-boroPro to not only inhibit fibroblast activation protein DPP enzymatic activity in vitro, but also partially inhibit fibroblast activation protein DPP enzymatic activity in vivo. This is accompanied by a modest growth attenuation of HT-29 tumors that induce fibroblast activation protein expression in the surrounding stroma. This association between inhibition of fibroblast activation protein enzymatic activity in vivo and tumor growth attenuation is consistent with the fibroblast activation protein S624A transfection experiments reported here, and supports our hypothesis that inhibition of fibroblast activation protein enzymatic activity attenuates the invasive capabilities of tumors.

Given the ability of Val-boroPro to inhibit the enzymatic activity of both fibroblast activation protein and DPP IV, the relative contributions to tumor growth of dual inhibition of fibroblast activation protein/DPP IV as opposed to fibroblast
activation protein–specific effects are unclear. However, a number of lines of evidence suggest that the tumor attenuation effects of Val-boroPro are due to inhibition of fibroblast activation protein and not DPPIV: (a) the biological effects of Val-boroPro on fibroblast activation protein can be seen independent of DPPIV as shown in DPPIV-deficient mice (36); (b) although there is hetero-dimerization of fibroblast activation protein and DPPIV in human cells, these proteins are uncoupled in mouse cells (28); (c) protein extracts of treated and untreated HT-29 xenografts did not show significant protein levels of DPPIV by Western analysis (data not shown). However, it is possible that Val-boroPro affects other currently unrecognized proteases that participate in tumor growth, and may have biological effects distinct from fibroblast activation protein. Future plans include further delineation of biological effects of fibroblast activation protein–specific inhibition utilizing fibroblast activation protein–selective small molecule or monoclonal antibody inhibitors of fibroblast activation protein. The specificity of monoclonal antibodies for fibroblast activation protein may offer significant advantages in determining fibroblast activation protein’s specific contribution to tumor stromagenesis.

In summary, we have shown that fibroblast activation protein overexpression potentiates tumor growth, and abrogation of its enzymatic activity can attenuate fibroblast activation protein–driven tumor growth. This establishes the proof-of-principle that the enzymatic activity of fibroblast activation protein plays an important role in the promotion of tumor growth, and is an attractive target for therapeutics designed to alter fibroblast activation protein–induced tumor growth by targeting its enzymatic activity.

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