Human Carcinoma Cell Growth and Invasiveness Is Impaired by the Propeptide of the Ubiquitous Proprotein Convertase Furin

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Abstract

Furin, a potent proprotein convertase involved in activation of several cancer-related substrates, is synthesized as an inactive zymogen, thus minimizing the occurrence of premature enzymatic activity that would lead to inappropriate protein activation or degradation. This natural inhibitory mechanism is based on the presence of an inactivating prosegment at the NH2 terminal of the zymogen. After initial autocatalytic cleavage, the prosegment remains tightly associated with the convertase until it reaches the trans-Golgi network where the dissociation of the prosegment and activation of furin occurs. We hypothesized that the inhibitory properties of the prosegment of furin (ppFur) could be beneficial if ectopically expressed in tumor cells. Transfection of four human head and neck squamous cell carcinoma cell lines with the complete ppFur cDNA sequence (pIRES-EGFP-ppFur) or with the empty expression vector (pIRES-EGFP) was done. The inhibitory effect was evaluated using in vitro tumorigenicity, invasion, anchorage-independent growth in soft agar, and proliferation assays, as well as by investigating impairment of furin substrates processing. Following transfection of ppFur, a significant reduction in cell proliferation, tumorigenicity, and invasiveness was observed in vitro and in vivo. These biological changes are directly related to the inhibition of furin-mediated activation of crucial cancer-related substrates, such as membrane type 1 matrix metalloproteinase, transforming growth factor-β, insulin-like growth factor-2, insulin-like growth factor-1 receptor, and vascular endothelial growth factor-C. PpFur expression in head and neck squamous cell carcinoma cell lines showed a mechanistic link between furin inhibition, decreased substrate processing, cell proliferation, and invasive ability. These findings suggest that furin inhibition is a feasible approach to ameliorate and even abolish the malignant phenotype of various malignancies. (Cancer Res 2005; 65(10): 4162-71)

Introduction

Limited proteolysis of inactive precursors is a regulatory mechanism responsible for the generation of biologically active proteins. This process is carried out by subtilin and/or kexin-related enzymes known as proprotein convertases (PC; ref. 1), which recognize and process precursor proteins at the consensus motif RX/RKR (2). To date, nine mammalian Ca2+-dependent proteases have been identified, including furin, PC1/PC3, PC2, PC4, PACE4, PC5/PC6, PC7/LPC/PC8, SKI/S1P, and NARC-1/PCSK9 (3–5). Furin, a well-characterized proprotein convertase, activates most of its substrates in the secretory pathway compartments. Some of the furin-activated substrates have significant roles in cancer development. Among these, metalloproteinases, growth factors, growth factor receptors, and adhesion molecules—such as membrane type 1 matrix metalloproteinase (MT1-MMP), transforming growth factor-β (TGF-β), insulin-like growth factor-2 (IGF-2), insulin-like growth factor-1 receptor (IGF-1R), and vascular endothelial growth factor-C (VEGF-C)—are directly associated with tumor progression by either modulating the degradation of the extracellular matrix or by influencing cell adhesion/locomotion and growth (6–8).

Although furin is ubiquitously expressed at low levels in human tissues, it has been shown to be up-regulated in various cell lines and in several malignant tumors, including non–small cell lung carcinomas, breast tumors, head and neck squamous cell carcinomas (HNSCC), and glioblastomas (9–12).

The synthesis of most proprotein convertases as inactive zymogens provides cells with the means to regulate spatially and temporally their proteolytic activities, minimizing the occurrence of premature enzymatic activity that would lead to inappropriate protein degradation. The inhibitory mechanism involves the presence of an inactivating prosegment at the NH2 terminal of the zymogen. Furin prosegment is autocatalytically processed in the endoplasmic reticulum. This event is a prerequisite for the efficient furin egress from this compartment (13, 14). After the initial cleavage, the prosegment remains tightly associated with the convertase. When it reaches the trans-Golgi network, the increase in H+ and/or Ca2+ concentration triggers a secondary cleavage, resulting in the dissociation of the prosegment and activation of the enzyme (15). Furin processes precursors either at the trans-Golgi network or the cell surface. It mediates a wide range of processing events, which, in pathologic situations, may exacerbate a disease state (16). The requirement of furin for the processing of tumor progression–related substrates suggests that selective inhibitors of furin have potential as agents capable of reducing the malignant phenotype of some tumors. Previous attempts to inhibit the processing activity in vivo have included the use of irreversible chloromethylketone inhibitors (17) and reversible peptide inhibitors (18). Major limitations of these agents include either cell cytotoxicity and/or poor cellular permeability and targeting. Other approaches used recombinant-based inhibitors (19–22). These strategies are based on the expression of proteins that contain a furin-like recognition sequence (RXR) within the inhibitor binding region of either human α1-antitrypsin (22), α2-macroglobulin (21), proteinase-8 (20), or the turkey ovomucoid third domain (19). Although reasonably effective, the inability of these recombinant proteins to inhibit selectively.
furin and not other proprotein convertases could be problematic. cl-PDX (cl-anti-trypsin-Portland) is a competitive inhibitor of furin. The *in vivo* and *in vitro* studies reported by our group and others showed that the serpin cl-PDX could be a useful tool for inhibition of tumorigenesis (8, 12, 23).

Zhong et al. (24) showed for the first time that furin prosegment, expressed *ex vivo* as an independent domain, can act *in trans* to selectively and efficiently inhibit precursor maturation by intracellular furin convertase (IC50 = 4 nmol/L).

Here, we show a significant reduction in cell proliferation, tumorigenicity, and invasiveness after transfecting and over-expressing furin prosegment, the natural endogenous furin inhibitor, in four different malignant HNSCC cell lines.

Materials and Methods

**Cell lines.** HNSCC cell lines A253, Fadu, SCC15, and SCC71 were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in Eagle's MEM, Spinner modification (S-MEM) with the addition of 2 mmol/L L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). SCC15 is derived from a well-differentiated squamous cell carcinoma (SCC) of the tongue. A253 and SCC71 are derived from moderately differentiated SCC of the submaxillary gland and soft palate, respectively. Fadu is derived from a poorly differentiated SCC of the pharynx. *In vivo* invasion assays and s.c. tumorigenesis assays showed that SCC15 has a moderate *in vivo* growth pattern, whereas A253, Fadu, and SCC71 have a markedly invasive *in vivo* growth pattern (25).

**Subcloning of preprofurin.** The preprosegment of furin (pfFurin, 351-bp coding for amino acids 1-109) was amplified for 25 cycles by PCR as described elsewhere (24). The sense and antisense pairs of oligonucleotides for furin that contain 5’ HindIII and 3’ BamHI sites are as follows: 5’ AAGCTTGAAGCCCTTTG-3’ (primer sense); 5’ GAATGGATAC-3’ (primer antisense) for furin (150 bp product); 5’-GAGATACCCACTCTGAGAATGATGACT-3’ and 5’-GGATCCGGTGGGCGGGCAAGCCGACA-3’ (primer antisense) for furin (150 bp product); 5’-GAGATACCCACTCTGAGAATGATGACT-3’ and 5’-GGATCCGGTGGGCGGGCAAGCCGACA-3’ (primer antisense) for furin (150 bp product); 5’-GGATCCGGTGGGCGGGCAAGCCGACA-3’ (primer antisense) for furin (150 bp product); 5’-GGATCCGGTGGGCGGGCAAGCCGACA-3’ (primer antisense) for furin (150 bp product); 5’-GGATCCGGTGGGCGGGCAAGCCGACA-3’ (primer antisense) for furin (150 bp product). 

**Transfection of cell lines.** Cells were stably transfected with either pRc/RS-EGFP vector (BD Biosciences Clontech, Palo Alto, CA) for stable transfection.

**Protein sample preparation and Western blot analysis.** For cell lysates, 70% to 80% confluent cells were washed thrice with cold PBS buffer. Four hundred microliters of lysis buffer (1 × PBS, 0.1% SDS, 0.5% Na deoxycholate, 1% Nonidet P-40), with the protease inhibitors 1 mmol/L aprotinin, 100 mmol/L phenylmethylsulfonyl fluoride, and 100 mmol/L Na3VO4, were added to the cultures that were incubated at 4°C for 15 minutes. Cells were scraped, passed through a 21-G needle, and incubated at 4°C for 30 minutes. Cell lysates were centrifuged at 10,000 g for 5 minutes and the supernatants were separated. Thirty micrograms of the cell lysates were fractionated by electrophoresis in 4% to 20% SDS-PAGE for pfFur expression analysis. Fifty micrograms of the cell lysates were submitted to electrophoresis to assess furin, IGF-1R, MT1-MMP, and actin expression.

**In vitro invasion assay.** The invasive ability of cells was assessed with Biocoat Matrigel invasion chambers (Becton Dickinson). Briefly, cells were trypsinized and 50,000 cells were suspended in serum-free S-MEM with 10% FBS, 0.1% SDS, 0.5% Na deoxycholate, 1% Nonidet P-40, and 0.1% bovine serum albumin. Medium containing 5% serum was used as a chemoattractant. Incubation was done for 24 hours. Cells that degraded the reconstituted extracellular matrix and passed through the invasion chamber were fixed, stained, and counted. A total of 18 filters (three per transfected cell line) were counted (an average of 1,000-1,200 cells per transfectant).

**Metalloproteinase gelatinase assay.** To evaluate gelatinase activity, we used the MMP Gelatinase Activity Assay kit (Chemicon International Inc., Temecula, CA) according to manufacturer's instructions. Briefly, cells were trypsinized and 50,000 cells were suspended in serum-free S-MEM with 0.1% bovine serum albumin. Medium containing 5% serum was used as a chemoattractant. Incubation was done for 24 hours. Cells that degraded the reconstituted extracellular matrix and passed through the invasion chamber were fixed, stained, and counted. A total of 18 filters (three per transfected cell line) were counted (an average of 1,000-1,200 cells per transfectant).

**Extracellular proteins (VEGF-C and TGF-β1) were assessed on 24-hour-conditioned medium concentrated 50-fold by filtration (Centriprep Microcon YM-10, Millipore, Billerica, MA). Proteins were electroblotted onto a nitrocellulose membrane and probed with monoclonal antibody against the Cys-rich region of furin (MON-152, Alexis, San Diego, CA), the TGF-β1 affinity-purified goat anti-human latent associated peptide IgG (R&D, Minneapolis, MN), IGF-1R (c-19, Santa Cruz Biotechnology, Santa Cruz, CA), MT-1MMP (mouse monoclonal anti-human IgG, kindly supplied by Dr. A.G. Arroyo, Unidad de Biología Molecular, Hospital Universitario de la Princesa, Madrid, Spain), ppFur (rabbit polyclonal anti-human IgG, developed by Dr. N.G. Seidah), and VEGF-C (H-190; Santa Cruz Biotechnology). Loading controls were done using an α-actin polyclonal antibody (1-19, Santa Cruz Biotechnology). Immunocomplexes were revealed with enhanced chemiluminescence based on the use of peroxidase-labeled IgG (Amer sham Biosciences, Piscataway, NJ). Western analysis of tumors derived from both ppFur-transfected cells or VA-transfected cells was done following similar procedures.

The bands corresponding to MT1-MMP, IGF-1R, TGF-β1, and VEGF-C were quantitated by densitometry using the NIH 1.61 program and the results were expressed as percentage of inhibition.

**Analysis of furin and PC5 mRNA expression by reverse transcription-PCR.** Total RNA from the VA and ppFur-transfected cell lines was extracted using TRIzol (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. RNA was reversed-transcribed using the Super Script one-step reverse transcription-PCR (RT-PCR) with Platinum Taq (Invitrogen) following manufacturer's instructions. The mixture was sequentially incubated for 30 minutes at 50°C and for 2 minutes at 94°C. PCR amplification of cDNAs used the oligonucleotides 5’-CAGCGGGTGGGCAACACTGTGCT-3’ (primer sense) and 5’-GGCAGGGTGGTACGACA-3’ (primer antisense) for furin (150 bp product); 5’-TGGACACTCTCTAGAAATGGATAC-3’ and 5’-GAGATACCCACTTAGGGGGACG-3’ for PC5/B (112 bp product; developed by Dr. N.G. Seidah); and 5’-GAGCAGAGGGCTTGCTGTCCTTTG-3’ and 5’-CATACATGCCGGAGGCGGTTG-3’ for actin (450 bp product). The conditions for furin and actin PCR amplification reactions were as follows: 15 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 70°C for 35 cycles. The conditions for PC5/B PCR amplification reaction were as follows: 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C for 40 cycles. The quantity of the total RNA introduced in the reverse transcription reaction was set independently for each gene product to be below the saturation point of the reaction. Amplified PCR products were analyzed on a 1.5% agarose gel. As a positive control for furin and PC5 mRNA expression, SCC15 was transfected with furin cDNA (26) and PC5 cDNA.

**In vivo tumorigenesis assay.** Mouse models (ppFur- or VA-transfected cells (5 × 106 VA- and ppFur-transfected cells for each cell line in 100 mm plates and incubated overnight in serum-free S-MEM. Conditioned medium was collected and concentrated 60 times down to 50 μL. Values are reported as MIP activity (percentage of control).**

**In vivo tumorigenesis assay.** While either ppFur- or VA-transfected cells (5 × 106) were injected into the s.c. tissue of severe combined immunodeficient (SCID) mice (five mice per cell line). To minimize animal-to-animal variations, we inoculated the VA-transfected cells and the ppFur-transfected cells in the right flank and in the left flank of each mice, respectively. Mice were shaved weekly to assess time of tumor development, appearance, and volume. Tumors were measured weekly
after the emergence of the first tumor for the pair of transfected cells by using a Vernier caliper. Volume (V) of the tumors was obtained by using the following equation: $V = \frac{(L_1 + L_2)}{2} \times L_1 \times L_2 \times 0.526$, where $L_1$ and $L_2$ are the length and width of the s.c. tumor.

All experiments using animals were done under Fox Chase Cancer Center Laboratory Animal Facility guidelines. All animals were killed by CO$_2$ inhalation.

### Immunochemistry

PpFur immunohistochemistry of tumors retrieved from mice inoculated in the dorsal s.c. tissue with the VA- and ppFur-transfected cells was done using paraffin-embedded material. After rehydration, sections were subjected to antigen retrieval by microwave boiling at low setting in distilled water for 10 minutes. A rabbit polyclonal antihuman PpFur IgG was used as primary antibody at 1/50 dilution to reveal preprosegment furin convertase.

CD31 immunohistochemistry of tumors retrieved from mice s.c. inoculated with VA- and ppFur-transfected cells was done using paraffin-embedded tissue. After rehydration, sections were subjected to antigen retrieval by microwave boiling at low setting in distilled water for 10 minutes. A rabbit polyclonal antihuman CD31 IgG was used as primary antibody at 1/50 dilution. An avidin-biotin-peroxidase kit (BioGenex, San Ramon, CA) was then used followed by the chromagen 3'-diaminobenzidine to develop the immunostain. Negative controls, not incubated with primary antibody, were used. All sections were counterstained with hematoxylin, mounted, and analyzed.

### Microvessel density determination

The vascular plexus surrounding and within peripheral compartment of the tumors (external 250-µm-wide areas of the tumors) were counted. An image analysis system (Fairfield Imaging, Nottingham, United Kingdom) was used to assist in the quantitation (0.104 mm$^2$ per field) of any brown-staining endothelial cell or endothelial cell cluster that was differentiable from adjacent vasculature, tumor cells, and other connective tissue elements; they were considered a single, countable microvessel based on the criteria of Weidner et al. (27). Vessel lumina were not necessary for a structure to be defined as a microvessels, and red cells were not used to define a vessel lumen. Five noncontiguous × 200 fields associated with the area of interest were randomly selected without bias toward areas of “highest neovascularization.” Microvessel density (MVD) was expressed as number of microvessels per 1,000 µm$^2$.

### Cell growth assay

$[^3]$H-thymidine incorporation was evaluated after plating 5 × 10$^4$ cells/well (ppFur and VA transfecants) in 96-well culture plates. Twenty-four hours after plating, cells were incubated in a growth arrest medium (0.5% PBS-S-MEM, t-Glu, Pen-Strep) for 24 hours. For the last 2 hours of incubation, 1 µCi/well $[^3]$H-methylthymidine (Moravek Biochemicals, Brea, CA) was added. Afterward, cells were washed twice with PBS and treated with 10% trichloroacetic acid at 4°C for 30 minutes and washed twice with water. Cells were then rinsed with 70% ethanol and air dried. Labeled DNA was dissolved with 200 µL 10 mmol/L NaOH, 1% SDS, and counted with Scintiverse (Fisher, Pittsburgh, PA). Results are expressed as percent with respect to the VA-transfected cells.

### Anchorage-independent growth in soft agar

To assess anchorage dependency of growth, 4 × 10$^5$ cells were suspended in complete medium containing 0.3% agar and seeded in triplicate in six-well plates onto a base layer of complete medium containing 0.5% agar. Cells were incubated at 37°C in 5% CO$_2$ in air. Complete medium was added every 3 days. After 14 days, colonies >100 µm in diameter were counted by inverted microscopy.

### Statistical analysis

Values are expressed as means with 95% confidence intervals. Tumor growth curves were constructed from the mean tumor volume at each point of measurement, with error bars representing 95% confidence intervals of the mean. ANOVA and log-rank test were used to assess differences in growth rate and time of tumor onset, respectively. For the *in vitro* invasion assay, cell proliferation assay, anchorage-independent growth in soft agar, and MMP-gelatinase activity assay, two-sided t test was used to compare differences between ppFur and control VA transfecants.

Differences with P < 0.05 were considered statistically significant. Unless otherwise stated in the text, each experiment was repeated twice.

### Results

**Furin expression and preprosegment of furin transfection.** Four HNSCC cell lines were selected for these studies because of their different patterns of invasiveness in *in vivo* invasion and s.c. tumorigenesis assays (25). In a previous study, we showed that expression of endogenous furin correlated with the aggressiveness of HNSCC cell lines (11). In this report, we showed that A253 and Fadu, the most aggressive cell lines, exhibited the highest levels of furin protein. SCC15, the least aggressive of the four cell lines analyzed, showed the lowest furin expression. SCC71, characterized by moderate invasiveness, showed an intermediate furin expression level (Fig. 1A).

The expression of furin preprosegment in the transfected cell lines was confirmed by Western analysis of HNSCC lysates (Fig. 1B). No ppFur could be detected in the extracellular medium and ppFur transfection did not affect cell survival (data not shown).

The expression of furin in the transfected cell lines was assessed by Western analysis of HNSCC lysates (Fig. 1C). This band showed a molecular weight of 90 kDa that may correspond to the active form of the enzyme rather than its pro-form (100 kDa). Furin expression was slightly higher in transfected cells, indicating to some extent a possible accumulation of the enzyme in the secretory pathway. There was no evident shift in the molecular weight of the protein, indicating that overexpression of presegegment did not result in the inhibition of autocatalytic cleavage of furin in the endoplasmic reticulum.

The prosegment of furin is a selective inhibitor of the parent enzyme. Nevertheless, Zhong et al. (24) showed *in vitro* that ppFur inhibits PCS at 10-fold lower concentrations than it inhibits furin. The authors also showed that ppFur inhibition of other proprotein convertases is negligible. To rule out the possibility that the effects caused by the prosegment of furin could be due to the inhibition of PCS as well as furin, we analyzed PC5 alongside furin expression via RT-PCR. We found as expected that furin is expressed in the four VA- and ppFur-transfected SCC cell lines. On the other hand, there was no detectable PC5/A expression in the cell lines analyzed (Fig. 1D).

**Preprosegment of furin effects on proliferative ability of head and neck squamous cell carcinoma cell lines.** PpFur inhibition of furin may have effects on cell proliferation. A functional IGF-1R is required for cell growth and survival in *in vitro* and *in vivo* of various transformed cells (28, 29). IGF-1R pro-form (∼200 kDa) is processed at the RKRB$^{740}$ site into the α-subunits and membrane-bound β-subunits (∼105 kDa). Western blot analysis showed that the VA-transfected cells expressed mostly the low molecular weight band corresponding to the processed form of the receptor. Conversely, in the ppFur-transfected cells, the form corresponding to the pro-form of the receptor (∼200 kDa) was more prominent than in the VA counterparts (Fig. 24). Densitometric values show that the processing of the growth factor was inhibited 27%, 30%, 10%, and 51% in A253, Fadu, SCC15, and SCC71, respectively.

TGF-β1 is another furin substrate that contributes to the decrease in invasiveness and tumorigenicity seen in the ppFur-transfected cell lines. In our system, we could detect TGF-β1 expression in tumor cell supernatants and found that ppFur expression has an inhibitory effect on its processing. Figure 2B shows that the processing of the pro-form of this growth factor is blocked in the ppFur-transfected cells. Densitometric values show that the processing of the growth factor was inhibited almost 100% in all of the cell lines.
Because IGF-1R processing regulates cell growth, we assessed the ability of ppFur-transfected cells to incorporate [3H]thymidine compared with control cell lines. Measurement of [3H]thymidine incorporation indicated that after ppFur transfection, a reduction (20-30%) in the proliferation rate was achieved in agreement with the inhibition of IGF-1R processing observed (Fig. 2C).

Colony formation in soft agar. The ability to form colonies in soft agar is a feature associated with malignant transformation. During this multistep process, cells become anchorage independent. Testing for this characteristic change is widely used to evaluate the malignant potential of cells in vitro (30). We investigated the effects of ppFur overexpression on anchorage-independent growth in soft agar. The results showed that the ppFur-transfected cell lines exhibited an ~50% reduction in their anchorage-independent growth compared with the VA-transfected counterparts (Fig. 3).

Preprosegment of furin effects on the invasive potential of head and neck squamous cell carcinoma cell lines. To assess ppFur effects on invasiveness, in vitro assays were done. The in vitro assay evaluated the invasive ability of ppFur and control VA transfectants by counting percentage of cells that degraded and passed through the Matrigel-coated inserts. The results showed that the ppFur-transfected cells exhibited ~50% less invasive than the VA-transfected counterparts (P < 0.05; Fig. 4C).

MT1-MMP, a well-known furin substrate, is a membrane-bound metalloproteinase implicated in the degradation of the
basement membrane and directly related to tumor cell invasiveness. Western analysis indicated that ppFur expression resulted in the impairment in MT1-MMP activation (Fig. 4A), in which the band corresponding to the pro-form (63 kDa) was remarkable more prominent than the activated form (60 kDa; Fig. 4A, arrowhead). Conversely, in the VA-transfected cells, the band corresponding to the mature form of the enzyme was practically the only detectable band.

Because MT1-MMP activation results in activation of progelatinase A (MMP-2; ref. 31), an enzyme that directly degrades collagen IV, the gelatinolytic potential of the transfectants was evaluated. Gelatinase activity was consistently and significantly inhibited in the ppFur-transfected cells (Fig. 4B), which explains the dramatic inhibitory effects of ppFur transfection on cell invasiveness.

Preprosegment of furin effects on the tumorigenic potential of head and neck squamous cell carcinoma cell lines. The tumorigenic ability of the transfectants were evaluated in s.c. xenografts using SCID mice. ANOVA and log-rank test indicated statistically significant differences in growth rate and time of tumor onset, respectively (P < 0.05; Fig. 5A-D). SCC71 cells showed the most striking difference in growth pattern, whereas the ppFur-transfected cells produced no or very small tumors and the VA-transfected cells gave rise to relatively large tumors. The latency time to tumor detection was longer for ppFur-transfected cells. The least difference in tumor volume was observed in SCC15 ppFur-transfected cells when compared with the VA-transfected counterparts. This agrees with the fact that this cell line expressed the lowest level of furin.

Animals bearing VA-derived tumors (right flank) and tumors derived from ppFur-transfected cells (left flank) were sacrificed at the end of the experiment. PpFur immunohistochemistry done using tumors derived from the all transfectant cell lines showed that ppFur was expressed (data shown corresponds to tumors developed after s.c. injection of VA- and ppFur-transfected SCC71, respectively; Fig. 5F and G).

Our results indicate that following transfection of ppFur cDNA into the HNSCC cell lines, a significant reduction in cell proliferation and invasiveness was observed in our in vitro studies. We hypothesize that the reduced tumor burden in the in vivo experiment is due in part to an inhibition of the proproliferative and proinvasive furin substrates IGF-1R and MT1-MMP, respectively. For that purpose, tumors developed from the cell lines that...
gave rise to the largest (SCC71) and smallest (SCC15) differences in tumor size between VA- and ppFur-transfected cells were chosen. Western analysis from tumor samples indicated that ppFur expression resulted in a partial impairment in MT1-MMP activation (Fig. 5E), in which the band corresponding to the pro-form (63 kDa) could be detected. The densitometric values show that the percentage of MT1-MMP processing inhibition was 26% and 47% for SCC15 and SCC71, respectively. Conversely, in the tumors derived from VA-transfected cells, the band corresponding to the mature form of the enzyme was the most prominent (60 kDa; Fig. 5E, arrowhead). Similarly, Western blot analysis of IGF-1R showed that the tumors derived from VA-transfected cells expressed mostly the low molecular weight band corresponding to the processed form of the receptor. Conversely, in the ppFur-transfected cells, the form corresponding to the pro-form of the receptor (200 kDa) was more prominent than in the VA counterparts (Fig. 5E). Densitometric vales show that the percentage of IGF-1R inhibition was 22% and 85% for SCC15 and SCC71, respectively.

Preprosegment of furin effects on the angiogenic potential of head and neck squamous cell carcinoma cell lines. Human malignancies are characterized by containing a mixture of VEGF overexpressing tumor cells and VEGF receptor overexpressing tumor-associated vessels. Among the several vascular cell growth factors, VEGF-C has been identified as having a significant role in the lymphatic propagation of HNSCCs (32, 33). Western analysis indicated that ppFur overexpression resulted in the inhibition in VEGF-C activation (Fig. 6A), in which the band corresponding to the pro-form (59 kDa) could be detected, whereas in the VA-transfected cells the band corresponding to the active form (~29 kDa) of the enzyme was practically the only one detected. Densitometric values show that the percentage of VEGF-C inhibition was 49%, 37%, 7%, and 73% for A253, Fadu, SCC15, and SCC71, respectively.

The MVD of the tumors developed from VA- and ppFur-transfected cells injected s.c. in mice was measured. Tumors derived from cell lines that showed the biggest (SCC71) and smallest (SCC15) difference in tumor size between VA- and ppFur-transfected cells were chosen. The tumors were stained with an antibody against CD31, an endothelial cell marker that is involved in endothelial cell-to-cell adhesion and leukocyte transmigration. Indeed, tumors derived from VA-transfected cells showed extensive CD31 staining of vessels (Fig. 6B1). Sections from ppFur-transfected cell–derived tumors exhibited a marked decrease in the CD31 staining pattern (Fig. 6B2). Quantification of vascularization in tumor sections for each transfected cell line (data highlighted in Fig. 6B) revealed a statistically significant decreased MVD in tumors derived from ppFur-transfected cells (P < 0.01).

Discussion

Furin, the most relevant proprotein convertase associated with human tumor progression, is physiologically expressed at low levels...
in several normal human tissues. Conversely, this proprotein convertase is overexpressed in several primary malignancies (9, 10). Our laboratory has shown that furin expression correlates with the degree of invasiveness of HNSCC cell lines (11). In the same study, we found that nonmetastasizing HNSCCs expressed significantly lower levels of furin protein than metastasizing carcinomas. Furthermore, we reported that ectopic overexpression of furin causes a significant increase in the invasive potential of human HNSCC cell lines of low and moderate aggressive potential in vitro and in vivo (34).

Proprotein convertases in general and furin in particular activate growth factors and growth factor receptors involved directly or indirectly in the acquisition of a more aggressive phenotype during tumor progression, such as TGF-β, IGF-2, IGF-1Rβ, and VEGF as well as MMPs (6–8, 35).

In this report, we used HNSCC cell lines A253, Fadu, SCC15, and SCC71, which are known to exhibit an invasive growth behavior when s.c. xenotransplanted into nude mice. SCC15, the least aggressive cell line, showed the lowest levels of furin expression. Conversely, A253, Fadu, and SCC71 cells were more invasive. A253 and Fadu, the most aggressive cell lines, showed the highest levels of furin expression when analyzed by Western blotting. SCC71 showed an intermediate furin expression in accordance with its intermediate aggressiveness. Another proprotein convertase, PC5, exists in two different isoforms, a soluble PC5A sorted to regulated secretory granules and a membrane-bound PC5B cycling between the trans-Golgi network and the cell surface. A previous in vitro study has shown that ppFur inhibits PC5 ~10-fold better (IC50 value) than furin (24). The possibility of ppFur inhibiting PC5A/B as well as furin was a concern in our cell system. To our knowledge, there is no report in which PC5A/B expression in HNSCC cell lines was studied. Thus, we assessed PC5 expression in the VA- and ppFur-transfected cell lines. RT-PCR analysis revealed that PC5A/B is not expressed in the HNSCC cell lines. Conversely, furin mRNA and protein was expressed in the same cell lines.

Previous reports showed that in different tumor cell systems, furin inhibition via α1-PDX overexpression resulted in a reduced invasiveness and in vivo tumorigenicity (8, 12, 23). Because the furin prosegment is the physiologic inhibitor of furin activation, at least during portions of the intracellular trafficking of the proprotein convertase, we decided to test whether the prosegment could act as an inhibitor of cancer cell growth and invasiveness. Apart from being essential for the proper folding of its cognate enzyme, furin prosegment is a potent and specific endogenous inhibitor of the parent enzyme and deters it from gaining premature activity in the inappropriate cellular compartments (24).

In the present report, we show that overexpression of furin prosegment decreases and even abolishes the invasive and proliferative ability of four different malignant HNSCC cell lines and that these biological effects parallel the inhibition of the activation of crucial proteases, growth factors, and growth factor receptors involved in these processes.
The \textit{in vitro} invasive behavior was significantly reduced (>50\%) when compared with the control counterparts after ppFur transfection of the four HNSCC cell lines. A253 and SCC71, two aggressive cell lines, showed the lowest level of invasiveness after transfection with furin prosegment. SCC15 had the lowest level of furin expression of the HNSCC cell lines studied. This correlates with the less dramatic reduction in the \textit{in vitro} invasive ability. The ability to degrade the extracellular matrix, allowing the malignant cells to reach the general circulation, thus increasing the chances for metastasis (36), is one of the most important processes involved in the gradual acquisition of the malignant phenotype during tumor progression. Several enzymes are induced and activated during extracellular matrix degradation. Among the enzymes involved in extracellular matrix degradation, furin plays a role by activating MT1-MMP. This membrane-type MMP is overexpressed in many SCCs and the expression levels found correlated positively with the overall malignancy of tumors (37). The expression of MT1-MMP was observed in the ppFur-transfected cells by Western analysis. PpFur-transfected cells exhibited a remarkable accumulation of the unprocessed form of VEGF-C processing and its direct consequence, the diminished type IV collagenase. Because in both the \textit{in vivo} and \textit{in vitro} systems and the \textit{in vitro} invasion assay the tumor cells are in contact with extracellular matrix components, comparing the \textit{in vivo} inhibition of processing (around 50\%) with the inhibition of cell invasiveness is more relevant and shows a better correspondence. Although MT1-MMP has some extracellular matrix-degradative ability by itself, it is the key enzyme in the activation of Pro-MMP-2, a potent type IV collagenase. Our results show that the impairment in MT1-MMP processing and its direct consequence, the diminished type IV collagenase (gelatinase) activation, is the main factor behind the drastic ppFur effects on tumor cell invasiveness. Our study indicates that cell lines with higher type IV collagenase inhibition also show diminished \textit{in vitro} invasive ability after ppFur transfection. Inhibition of MT1-MMP activation is not the sole mechanism responsible for effects of ppFur transfection. Indeed, other substrates may contribute to the final biological effect as shown herein by inhibition of growth factors and growth factor receptors. An amplifying effect of the cascade from furin to cell invasiveness could be due to other factors that are put into play by extracellular matrix signaling as shown in our case by the differences between \textit{in vivo} and \textit{in vitro} MT1-MMP activation levels that showed a higher inhibition \textit{in vivo}.

TGF-\(\beta\) is known for its role in enhancing invasion and metastasis in several human and experimental cancer models (38). We have shown a significant inhibition of TGF-\(\beta\)1 activation in ppFur-transfected cell lines. Activation of IGF-1R and TGF-\(\beta\)1 are known to up-regulate cell proliferation in several cell types. It is well known that a functional IGF-1R is required for cell growth and plays a crucial role in survival of various transformed cells \textit{in vitro} and \textit{in vivo} (28). Defects in IGF-1R expression and/or activation inhibit tumorigenicity and reverse the transformed phenotype (39). The decrease in the processed IGF-1R shown by Western analysis resulted in a reduced cell proliferation activity as determined by the lower counts in the \(\beta\)H]thymidine incorporation. Similarly, the most striking differences were found in the most aggressive cell lines.

<table>
<thead>
<tr>
<th>A253</th>
<th>Fadu</th>
<th>SCC15</th>
<th>SCC71</th>
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<tbody>
<tr>
<td>VA</td>
<td>ppFur</td>
<td>VA</td>
<td>ppFur</td>
</tr>
<tr>
<td>51.5 KDa</td>
<td>35.5 KDa</td>
<td>28.7 KDa</td>
<td>Pro-VEGF-C</td>
</tr>
<tr>
<td>51.5 KDa</td>
<td>35.5 KDa</td>
<td>28.7 KDa</td>
<td>VEGF-C</td>
</tr>
<tr>
<td>SCC15</td>
<td>13.6±2.02</td>
<td>7.57±0.75</td>
<td></td>
</tr>
<tr>
<td>SCC71</td>
<td>19.5±1.21</td>
<td>2.3±0.14</td>
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</tr>
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</table>

\(P < 0.01\).
Overexpression and/or constitutive activation of IGF-1R in a variety of cell types leads to ligand-dependent growth in serum-free medium and to the establishment of a transformed phenotype, such as the ability to form colonies in soft agar (40, 41). Sell et al. (42) were the first to show that the expression of IGF-1R in IGF-1R−/− cells restored their ability to grow in soft agar. In this report, we show that ppFur inhibition of IGF-1R processing leads to a significant reduction in the number of colonies formed in soft agar.

Angiogenesis, the growth of new capillaries from preexisting blood vessels, is essential for cancer to grow beyond minimal size and metastasize (43). VEGF is regarded as the major angiogenesis factor during epithelial carcinogenesis in a large number of malignant human cancers and in tumor metastases (44, 45). Among the five members of the VEGF family, VEGF-C is the only bona fide furin substrate. VEGF-C expression has been detected in 50% of all the human cancers analyzed, and its expression has been correlated with lymph node dissemination in human cancers (46). VEGF-C plays a significant role by enhancing the neoformation of lymphatic and blood vessels in several human malignant diseases, including cancer of the head and neck (47). A recent study from our laboratory has shown that there is a close association between progressive squamous carcinogenesis in the human tongue, epithelial furin expression, VEGF-C expression, and neovascularization (48). This suggests that furin may promote tumor-associated angiogenesis through enhanced VEGF-C processing.

The fact that VEGF-C is processed by furin at the Q-VHSIIIR SLPI20 consensus motif (35) prompted us to examine the pattern of expression of VEGF-C after ppFur transfection. Our results show that pro-VEGF-C processing is blocked by the inhibitory prosegment of furin. The VA-transfected cell lines totally processed the VEGF-C pro-form. We evaluated the in vivo angiogenic potential of the transfectants in s.c. xenografts using SCID mice. MVD values were significantly lower in the tumors derived from ppFur-transfected cell lines than in tumors derived from VA-transfected cells.

In summary, this report describes a functional link between inhibition of substrate precursor processing achieved by furin and the extent of in vitro and in vivo significant reduction in cell proliferation, tumorigenicity, angiogenesis, and invasiveness after furin prosegment transfection of HNSCC cell lines. These biological changes are directly related to the inhibition of furin-mediated activation of crucial cancer-related substrates, such as MT1-MMP, TGF-β1, IGF-1R, and VEGF-C. These experiments highlight that in vivo tumor growth inhibition in this experimental system is due to an impairment in furin-mediated activation of cancer-related substrates. These findings suggest that furin inhibition is a feasible approach to ameliorate and even abolish the malignant phenotype of various malignancies. In this sense, ppFur inhibition of substrate activation is almost never complete. Consequently, the physiologic low levels of furin expression are maintained. These baseline findings may be useful in providing the basis for future development of furin prosegment–based therapies for cancer growth inhibition and at the same time preserving normal furin activity in noncancer cells.

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