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Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism

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Abstract

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Background and objectives—Cholangiocarcinoma is a devastating cancer of biliary origin with limited treatment options. The growth factor, progranulin, is overexpressed in a number of tumours. The study aims were to assess the expression of progranulin in cholangiocarcinoma and to determine its effects on tumour growth.

Methods—The expression and secretion of progranulin were evaluated in multiple cholangiocarcinoma cell lines and in clinical samples from patients with cholangiocarcinoma. The role of interleukin 6 (IL-6)-mediated signalling in the expression of progranulin was assessed using a combination of specific inhibitors and shRNA knockdown techniques. The effect of progranulin on proliferation and Akt activation and subsequent effects of FOXO1 phosphorylation were assessed in vitro. Progranulin knockdown cell lines were established, and the effects on cholangiocarcinoma growth were determined.

Results—Progranulin expression and secretion were upregulated in cholangiocarcinoma cell lines and tissue, which were in part via IL-6-mediated activation of the ERK1/2/RSK1/C/EBP β pathway. Blocking any of these signalling molecules, by either pharmacological inhibitors or shRNA, prevented the IL-6-dependent activation of progranulin expression. Treatment of cholangiocarcinoma cells with recombinant progranulin increased cell proliferation in vitro by a mechanism involving Akt phosphorylation leading to phosphorylation and nuclear extrusion of FOXO1. Knockdown of progranulin expression in cholangiocarcinoma cells decreased the expression of proliferating cellular nuclear antigen, a marker of proliferative capacity, and slowed tumour growth in vivo.

Conclusions—Evidence is presented for a role for progranulin as a novel growth factor regulating cholangiocarcinoma growth. Specific targeting of progranulin may represent an alternative for the development of therapeutic strategies.

INTRODUCTION

Cholangiocarcinomas are devastating cancers of intrahepatic and extrahepatic biliary origin, which are increasing in both worldwide incidence and mortality.¹² Conventional chemotherapy and radiation therapies are not effective in prolonging long-term survival¹; therefore it is important to understand the cellular mechanisms of cholangiocarcinoma cell growth with a view to developing novel chemopreventive strategies.

Progranulin (PGRN) is a secreted glycoprotein that mediates cell cycle progression and cell motility.³ It activates Akt and extracellular signal-regulated kinase (ERK) signalling cascades, among others.^{4–6} The precise mechanism of action of PGRN is unknown. PGRN is highly expressed in aggressive cancer cell lines and clinical specimens including breast, ovarian and renal cancers.³ In the liver, PGRN has been identified as a therapeutic target for the treatment of hepatocellular carcinoma⁷; however, its involvement in the aetiology and progression of cholangiocarcinoma is unknown.

The association between chronic inflammation and the development and progression of malignancy is exemplified in the biliary tract, where persistent inflammation strongly predisposes individuals to cholangiocarcinoma.⁸⁹ The inflammatory cytokine, interleukin-6 (IL-6), enhances tumour growth in cholangiocarcinoma by altering gene expression via autocrine mechanisms.⁸⁹ Interaction of IL-6 with its receptor complex can lead to activation

of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway¹⁰¹¹ and the ERK1/2 mitogen-activated protein kinase (MAPK) pathway.¹²

Comparison of the 5' sequences of the genes for human and mouse PGRN revealed several putative binding sites that are conserved between the two species, including CCAAT-enhancer binding protein β (C/EBP β).¹³ C/EBP β is responsive to various Ca²⁺- and cAMP-dependent pathways that are stimulated by IL-6 signalling¹⁴; however, the role of IL-6-mediated C/EBP β activation in PGRN action is unknown.

One of the downstream effectors of ERK1/2 activation is ribosomal protein S6 kinase-1 (RSK1), which can phosphorylate many cytosolic and nuclear targets that regulate diverse cellular processes including cell proliferation, cell survival, cell growth and motility.¹⁵ RSK1 has been shown in particular to promote the survival of hepatic stellate cells by phosphorylating C/EBP β in response to carbon tetrachloride.¹⁶

In the present study, we show an increase in expression and secretion of PGRN from cholangiocarcinoma cells and clinical samples via an IL-6/ERK1/2, RSK1/C/EBP β -mediated mechanism. Furthermore, we demonstrate that PGRN has growth-promoting effects on cholangiocarcinoma via the Akt-dependent phosphorylation and nuclear extrusion of FOXO1, and that knocking down the expression of PGRN slows the growth of cholangiocarcinoma in vitro and in vivo.

MATERIALS AND METHODS

Cell lines

We used four human cholangiocarcinoma cell lines (Mz-ChA-1, HuCC-T1, CCLP1 and SG231) from different origins. Mz-ChA-1 cells, from human gallbladder,¹⁷ were a gift from Dr G Fitz (University of Texas Southwestern Medical Center, Dallas, Texas, USA). CCLP-1,¹⁸ HuCC-T1¹⁹ and SG231,²⁰ from intrahepatic bile ducts, were a gift from Dr A J Demetris (University of Pittsburgh, Pittsburgh, Pennsylvania, USA) and were cultured as described.^{18–20} The human immortalised non-malignant cholangiocyte cell line, H69 (from Dr G J Gores, Mayo Clinic, Rochester, Minnesota, USA), was cultured as described.²¹ In addition, the primary human intrahepatic cholangiocyte cell line (HIBEC) was purchased from ScienCell (Carlsbad, California, USA) and cultured according to the manufacturer's instructions.

Real time PCR

Real time PCR was performed as previously described²²²³ using commercially available primers designed against human PGRN, IL-6, C/EBP β , proliferating cellular nuclear antigen (PCNA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; SABioscience, Frederick, Maryland, USA). A $\Delta\Delta$ CT analysis was performed using untreated cells or the H69 cholangiocyte cell line as the control samples, as appropriate.²⁴ Data are expressed as mean relative mRNA levels $\pm$ SEM (n=4).

Immunoblotting

Immunoblots to detect PGRN and β -actin were performed as previously described²⁵ using specific antibodies against each protein. Data are expressed as fold change (mean $\pm$ SEM) in the relative expression after normalisation with β -actin.

For phospho-specific immunoblotting experiments, cells were treated with recombinant human IL-6 (rIL-6), in the presence or absence of ERK1/2 inhibitor, PD98059 (10 μ M²⁶), or the RSK inhibitor, SL-0101-1 (0.2 μ M²⁷) for the times indicated. Immunoblots were performed using antibodies against phospho-ERK1/2 (Cell Signaling Technology, Beverly, Massachusetts, USA), total ERK1/2 (Cell Signaling Technology), phospho-C/EBP β (Cell Signaling Technology) and total C/EBP β (Santa Cruz Biotechnology, Santa Cruz, California, USA). Data are expressed as fold change in band intensity (mean $\pm$ SEM) of the phospho-specific form of each protein after normalisation to the corresponding total protein.

Cholangiocarcinoma tissue array analysis

Immunoreactivity of PGRN was assessed in commercially available Accumax tissue arrays (Isu Abxis Co, Seoul, Korea) by immunohistochemistry as described.²⁸ Semi-quantitative analysis was performed by three independent observers in a blinded fashion, using the following parameters: staining intensity was assessed on a scale from 1 to 4 (1, no staining; 4, intense staining), and the abundance of positively stained cells was given a score from 1 to 5 (1, no cells stained; 5, 100% stained). The staining index was then calculated by multiplying the staining intensity by the staining abundance giving a range from 1 to 20.

PGRN secretion

Cells were suspended in Hank's-buffered saline solution (1×10^7 cells/ml) and then incubated for 6 h at 37°C. The amount of PGRN released into the medium was then assayed using a commercially available PGRN ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to the manufacturer's instructions.

In parallel, serum samples were obtained from patients with cholangiocarcinoma (n=22) and age-matched controls (n=25). Hepatic bile was also collected aseptically from T-tube drainage during postoperative day 1–3 from patients with intrahepatic stones or gallstones (n=25) or cholangiocarcinoma (n=22). Serum and bile samples were immediately frozen at -80°C until used for the evaluation of PGRN levels. PGRN content was assessed using enzyme immunoassay (EIA) kits as outlined above. The human serum and bile samples were obtained from a tissue bank with deidentified clinical samples (collected with the approval of the ethics committee of the IRCCS Istituto Clinico Humanitas) and were analysed in a coded fashion in the laboratory of PI.

Establishment of stable transfected cell lines

The role of IL-6 and C/EBP β expression in the upregulation of PGRN and the role of PGRN expression in cholangiocarcinoma growth were determined in cell lines where these genes were knocked down. These cells were established using SureSilencing shRNA plasmids for human IL-6, C/EBP β and PGRN respectively (SABiosciences), containing a marker for neomycin resistance for the selection of stably transfected cells following previously

described methodology.²³ The resulting cell lines were designated Mz-IL-6 shRNA, Mz-C/EBP β shRNA, Mz-PGRN shRNA and Mz-neo neg (mock-transfected control).

Activity assays

Rsk1—Cells were treated with recombinant human PGRN (rPGRN) at 1 μ g/ml in the absence or presence of PD98059 (10 μ M²⁶), for various times up to 24 h. After treatment, the cells were washed in phosphate buffered-saline (PBS), lysed at 1×10^7 cells/ml, and the assay and data analysis were performed according to the manufacturer's instructions (R&D Systems).

Akt and FOXO1—Mz-ChA-1 cells were treated with rPGRN at 1 μ g/ml for various times up to 24 h. After treatment, each well was washed in PBS and fixed in 100 μ l 4% formaldehyde in PBS for 20 min. Phosphorylation levels of Akt and FOXO1 were then assessed using commercially available ELISA kits (both from Active Motif, Carlsbad, California, USA), and data were analysed according to the manufacturer's instructions.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed on DNA–protein complexes isolated from Mz-IL-6 shRNA cells and Mz-Neo neg cells. Cells were fixed in 1% (v/v) formaldehyde in cell culture medium and lysed in the lysis buffer provided in the ChIP-IT Express immunoprecipitation kit (Active Motif). DNA was then sheared using the MseI restriction enzyme at 37°C for 30 min. DNA fragments bound to C/EBP β were precipitated using a specific C/EBP β antibody previously used for chromatin immunoprecipitation (Santa Cruz Biotechnology²⁹) according to the manufacturer's instructions. The relative amount of PGRN promoter precipitated was assessed by real time PCR using specific primers designed around the –530 region of the PGRN promoter (SABiosciences). A Δ CT analysis was performed²⁴ using the amount of PGRN promoter in the input DNA for normalisation.

Cell proliferation assays

Cell lines were stimulated with rPGRN (0.25–1 μ g/ml) for 48 h. In other experiments, cells were pretreated with the Akt inhibitor, FPA124 (1 μ M³⁰), for 1 h before the addition of rPGRN (1 μ g/ml). Cell proliferation was assessed using a colorimetric MTS cell proliferation assay (CellTiter 96 AQueous; Promega Corp, Madison, Wisconsin, USA), and absorbance was measured at 490 nm with a microplate spectrophotometer (Versamax; Molecular Devices, Sunnyvale, California, USA). In parallel, Mz-ChA-1 cells were plated in 96-well plates and allowed to adhere overnight. The number of cells was counted in three non-overlapping fields per well immediately before the addition of rPGRN (1 μ g/ml) and again 24 h after stimulation. A similar cell counting experiment was performed to compare the basal rate of proliferation in Mz-ChA-1, Mz-neo neg and Mz-PGRN shRNA cells. In all cases, data were expressed as the fold change in treated cells compared with vehicle-treated controls.

Treatment of nude mice

In vivo experiments were performed as described previously²³ using Mz-ChA-1 cells or Mz-PGRN shRNA to establish tumours. Briefly, 8-week-old male BALB/c nude (nu/nu) mice were kept in a temperature-controlled environment (20–22°C) with a 12 h light–dark cycle with free access to drinking water and standard mouse chow. Cells (5×10^6) were suspended in 0.25 ml extracellular matrix gel and injected subcutaneously into the left back flank of these animals. After tumour establishment (10 days), dimensions were measured twice a week with an electronic calliper, and volume determined as: tumour volume (mm^3) = $0.5 \times (\text{length (mm)} \times \text{width (mm)} \times \text{height (mm)})$. Tumour protein selectively expressed by cholangiocytes was evaluated in the resulting tumours after CK-7 immunohistochemical staining. PCNA immunoreactivity (a marker of proliferative capacity) and activated caspase 3 immunoreactivity (a marker of apoptosis) were also evaluated.

Statistical analysis

All data are expressed as mean $\pm$ SEM. For data exhibiting normal distribution, differences between two groups were analysed by the Student unpaired t test and analysis of variance when more than two groups were analysed, followed by an appropriate post hoc test. When the normality test failed, a Mann–Whitney U test was performed when two groups were compared, and a Kruskal–Wallis one-way analysis of variance by ranks was performed when three or more groups were compared. In each case, a p value of less than 0.05 was used to indicate significance.

RESULTS

PGRN expression and secretion is increased in cholangiocarcinoma

The expression of PGRN mRNA and protein was markedly upregulated in all cholangiocarcinoma cell lines studied compared with the non-malignant H69 and HIBEC cell lines (figure 1A; quantitative analysis of immunoblots not shown). Immunoblotting revealed a strong band at 68 kDa, which is consistent with the reported molecular mass of PGRN, and a weaker band at 88 kDa, which may correspond to the glycosylated form.³¹ Immunohistochemical analysis of human liver biopsy samples indicated that there is also increased PGRN immunoreactivity in cholangiocarcinoma samples compared with non-malignant controls (figure 1B). The increase in PGRN expression in cholangiocarcinoma cells was paralleled by an increased concentration of PGRN in the conditioned medium from these cells compared with the H69 or HIBEC cells (figure 1C). Furthermore, in a pilot study, increased PGRN levels could be detected in the serum (but not bile; not shown) from patients with cholangiocarcinoma compared with non-malignant controls (figure 1D).

PGRN is increased in cholangiocarcinoma by IL-6-dependent activation of the ERK1/2/RSK1/C/EBP β pathway

IL-6 is overexpressed in cholangiocarcinoma and shares a longstanding association with the neoplastic transformation of cholangiocytes to cholangiocarcinoma cells.⁸⁹ From this, we surmised that IL-6 signalling may be driving the increase in PGRN expression seen in cholangiocarcinoma. Treatment of Mz-ChA-1 cells with rIL-6 increased PGRN mRNA and

protein expression at 2 and 4 h after treatment (figure 2A, quantitative analysis of immunoblots not shown). Although significant, this effect was not particularly dramatic, probably because of the large endogenous IL-6 production from these cells.⁸⁹ An alternative strategy was to establish a stable IL-6 knockdown cell line. Stable transfection of IL-6 shRNA into Mz-ChA-1 cells decreased IL-6 expression in the resulting clone (Mz-IL-6 shRNA) by 96% of the control transfected cell line (Mz-neo neg; online supplemental figure S1). PGRN mRNA and protein expression were decreased in the Mz-IL-6 shRNA cells compared with the Mz-neo neg control cell line (figure 2B), providing further evidence that IL-6 may drive PGRN expression in cholangiocarcinoma.

To determine the possible molecular mechanism by which IL-6 can regulate PGRN expression, Mz-ChA-1 cells were treated with rIL-6 in the presence or absence of inhibitors of the ERK1/2 and RSK1 pathway. Once again, IL-6 increased the expression of PGRN, which could be blocked by inhibitors of ERK1/2 and RSK1 (figure 3A). Inhibition of the JAK/STAT pathway could not prevent the IL-6-mediated upregulation of PGRN expression (data not shown). Furthermore, treatment of Mz-ChA-1 cells with rIL-6 increased the phosphorylation, and hence activation, of ERK1/2 (figure 3B; online supplemental figure S2). The activity of RSK1 was also increased in Mz-ChA-1 cells after rIL-6 treatment, which could be attenuated by the specific inhibitor of the ERK1/2 pathway (figure 3C), suggesting that RSK1 activation is downstream of ERK1/2 activation in the IL-6-mediated signal-transduction cascade. In parallel, we assessed the activation state of these intermediary-signalling molecules in the Mz-IL-6 shRNA cell line. There was decreased basal ERK1/2 phosphorylation in the Mz-IL-6 shRNA compared with the Mz-neo neg cell line (figure 3D). In addition, there was a decrease in RSK1 activity in the Mz-IL-6 shRNA cell line compared with the Mz-neo neg cells (figure 3E), further supporting the concept that IL-6 may be increasing PGRN expression by activating a signal-transduction pathway involving ERK1/2-dependent activation of RSK1.

As mentioned above, one of the downstream targets of RSK1 is C/EBP β .¹⁶ IL-6 treatment increased the phosphorylation and hence activation of C/EBP β , which could be blocked by inhibitors of the ERK1/2 and RSK1 pathway (figure 4A). Furthermore, in Mz-IL-6 shRNA cells, there was decreased phosphorylation of C/EBP β (figure 4B). If our hypothesis that C/EBP β is controlling the IL-6-driven expression of PGRN in cholangiocarcinoma is correct, when we remove IL-6 signalling, there should be less C/EBP β bound to the PGRN gene promoter. Indeed, chromatin immunoprecipitation showed less C/EBP β bound to the PGRN promoter region in the Mz-IL6 shRNA cells than in the mock-transfected Mz-neo neg cells (figure 4C). To further assess if C/EBP β activation is responsible for the expression of PGRN, we established a stable knockdown cell line using a C/EBP β shRNA vector. C/EBP β mRNA and protein expression was knocked down to ~40% of the mock-transfected Mz-neo neg cell line (online supplemental figure S3). The basal expression of PGRN in this cell line was significantly less than that seen in Mz-Neo neg cells (online supplemental figure S3 and figure 4D). Furthermore, IL-6 treatment increased PGRN expression in the Mz-neo neg, but not the Mz-C/EBP β shRNA, cell line (figure 4D), indicating a role for C/EBP β in the IL-6-driven increase in PGRN expression in cholangiocarcinoma cells.

PGRN has growth-promoting effects on cholangiocarcinoma

To assess the consequences of increased PGRN expression, we treated our cholangiocarcinoma cell lines with rPGRN and assessed the effects on cell proliferation. In all cell lines studied, rPGRN caused a significant increase in cell proliferation (figure 5A, online supplemental figure S4), which could be blocked by pretreatment with a specific Akt inhibitor (figure 5B). Furthermore, treatment of Mz-ChA-1 cells with rPGRN increased pAkt (online supplemental figure S5).

One of the downstream consequences of Akt activation is the phosphorylation and nuclear extrusion of FOXO1.³² Indeed, treatment of Mz-ChA-1 cells with rPGRN increased the phosphorylation of FOXO1 as demonstrated by an EIA activity kit (figure 6A). With immunofluorescence microscopy, FOXO1 was predominantly observed in the nucleus under basal conditions, but was extruded into the cytoplasm after treatment with rPGRN (figure 6B). This nuclear extrusion was prevented by pretreatment of the cells with the Akt inhibitor, FPA124 (figure 6B).

To further define a role for PGRN in cell proliferation, we established stable transfected cell lines using a specific PGRN shRNA vector to knock down PGRN expression. PGRN expression was reduced to ~20% of the parental cell line (online supplemental figure S6). There was a significant decrease in the expression of PCNA (a marker of proliferative capacity; figure 7A) and a decrease in cell proliferation (as assessed by cell number after 24 h; figure 7B) in the Mz-PGRN shRNA cells compared with the mock-transfected Mz-neo neg cell line or the parental Mz-ChA-1 cells, suggesting that PGRN exerts a growth-promoting effect on cholangiocarcinoma cells. To assess whether this effect translates into a change in tumour growth in vivo, we used a xenograft model of cholangiocarcinoma. The tumours derived from the Mz-PGRN shRNA cell line were smaller initially (online supplemental figure S7A), and over the time course of the experiment (36 days), tumours derived from the Mz-ChA-1 cells increased ~500%, whereas tumours derived from the Mz-PGRN shRNA cells only increased 300% (figure 7C; online supplemental figure S7A), suggesting that decreasing PGRN expression or activity slows tumour growth and progression. Histological analysis of the xenografted tumours revealed that all cancer cells within both groups were CK-7 positive, indicating a cholangiocyte phenotype (online supplemental figure S7B). Furthermore, there were significantly fewer PCNA-positive nuclei in the tumours derived from the Mz-PGRN shRNA compared with the Mz-ChA-1-derived tumour (online supplemental figure S8A). However, there was little activated caspase 3 in either tumour type (online supplemental figure S8B), suggesting that PGRN exerts its effects mainly on proliferation rather than the apoptotic pathway.

DISCUSSION

The major findings of this study relate to the mechanism and consequences of increased PGRN expression in cholangiocarcinoma. We demonstrated that: (1) PGRN expression and secretion is increased in cholangiocarcinoma compared with non-malignant counterparts, via IL-6-driven activation of the ERK1/2/RSK1/C/EBP β pathway; and (2) PGRN exerts growth-promoting effects on cholangiocarcinoma cells via activation of Akt and subsequent phosphorylation and nuclear extrusion of FOXO1. These data suggest that the upregulation

of PGRN may be a key feature associated with the progression of cholangiocarcinoma and that inhibiting PGRN expression or function may be a viable target for the development of an effective adjunct therapy to treat this deadly disease.

Consistent with our observations that PGRN expression is upregulated in cholangiocarcinoma, increased PGRN expression and secretion has been found in a number of other tumour types.³⁻⁷³³ In experimental systems, PGRN confers an aggressive phenotype on poorly tumorigenic epithelial cancer cells.³ The malignancy of highly tumorigenic PGRN-expressing cell lines depends on the expression level, since attenuating PGRN mRNA levels greatly inhibits tumour progression.³ Furthermore, PGRN expression has recently been identified as a potential prognostic biomarker for predicting the progression-free and overall survival of patients with epithelial ovarian cancer, as patients with significantly higher PGRN levels display poorer prognosis.³³³⁴ Given its role in tumorigenesis, PGRN may prove a useful clinical target for both prognosis and therapy.

IL-6 production is dramatically upregulated in cholangiocarcinoma, which is consistent with the long-standing association between chronic inflammation and the neoplastic transformation of cholangiocytes.⁸⁹ It enhances cholangiocarcinoma growth by altering gene expression via the JAK/STAT¹⁰¹¹ or ERK1/2 MAPK¹² pathway in an autocrine manner.⁸⁹ In our hands, inhibitors of the JAK/STAT pathway did not prevent the effects of IL-6 on PGRN expression. Therefore the data presented here identify PGRN as yet another target of IL-6-mediated signalling via the activation of ERK1/2 MAPK, but not JAK/STAT. Downstream of IL-6-mediated ERK1/2 activation is the activation of RSK1. While RSK1 is a well-known downstream effector of ERK1/2 activation in other cell types,¹⁵ to our knowledge our data represent the first indication that RSK1 may be responsible for some IL-6-mediated events seen in cholangiocarcinoma.

As mentioned above, RSK1 has been shown to activate C/EBP β in hepatic stellate cells in an experimental model of liver fibrosis.¹⁶ We chose to focus on C/EBP β as a potential target of the IL-6-driven signalling pathway, as genetic analysis of the promoter region of the human and mouse PGRN gene has revealed a highly conserved region containing binding sites for C/EBP β .¹³ The data presented support previous findings that C/EBP β can be a molecular target of RSK1,¹⁶ but provide the first indication that RSK1 can phosphorylate and activate C/EBP β to mediate the IL-6 signalling events in cholangiocarcinoma. Specifically, more C/EBP β was found bound to the PGRN promoter in cells with high levels of IL-6 (Mz-neo neg cells) than in cells with IL-6 expression knocked down (Mz-IL-6 shRNA cells). Knocking down C/EBP β expression dampened PGRN expression and prevented the IL-6-driven upregulation of PGRN expression in cholangiocarcinoma.

PGRN acts as a growth factor in a number of other cancer cell types and is capable of activating a number of different signal-transduction pathways,³ although a PGRN receptor has not yet been identified. The data presented here indicate that PGRN exerts its proliferative effects on cholangiocarcinoma cells via the activation of Akt signalling. We focused on the Akt signalling pathway in this study, as it has previously been identified as a downstream modulator of the proliferative properties of a number of other growth factors in cholangiocarcinoma.³⁵⁻³⁸ For example, prostaglandin E2 promotes cholangiocarcinoma cell

growth and invasion through activation of the epidermal growth factor receptor and Akt.³⁵ Xu *et al*³⁶ clearly demonstrated that liver-specific disruption of Smad4 and PTEN led to the development of cholangiocarcinoma, which was associated with increased pAkt.³⁶ Conversely, a number of agents that have been identified as antiproliferative in cholangiocarcinoma exert their effects via the inhibition of Akt signalling.^{39–43} Taken together, these data support a role for Akt signalling in the upregulation of PGRN expression and subsequent initiation and progression of cholangiocarcinoma. However, a role for other transduction pathways in the proliferative effects of PGRN on cholangiocarcinoma cannot be ruled out and warrants further investigation.

In this study, we show that a downstream target of Akt is FOXO1 and that phosphorylation of FOXO1 causes inactivation and nuclear extrusion of this protein in cholangiocarcinoma cells. This is consistent with our current knowledge of how the FOXO transcription factor family is regulated.⁴⁴ FOXO1 is known to regulate the expression of genes that regulate apoptosis and growth arrest,⁴⁴ and agents that are known to slow the growth and progression of various tumours, such as resveratrol and camptothecin, do so through activation of FOXO1.^{45,46} Overactivation of Akt and subsequent suppression of FOXO-mediated transcription has also been demonstrated in a number of other tumours.^{47,48} Here we demonstrate that the downstream consequence of PGRN overexpression in cholangiocarcinoma cells is activation of Akt and subsequent nuclear extrusion of FOXO1 and suppression of its activity.

In conclusion, the data presented in this study indicate that PGRN expression and secretion is upregulated in cholangiocarcinoma as a result of IL-6-mediated activation of the ERK1/2 MAPK/RSK1/C/EBP β pathway. PGRN therefore has growth-promoting properties, which it exerts on surrounding cells via the activation of Akt and subsequent inhibition of FOXO1 transcriptional activity. A schematic diagram summarising our findings can be found online in supplemental figure S9. Taken together, our data indicate that PGRN, and its subsequent signal-transduction pathway, may be a viable option for developing adjunct therapies for treating cholangiocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance of this study

What is already known about this subject?

- ▶ Progranulin expression is increased in breast, ovarian and renal cancers.
- ▶ Progranulin expression correlates with tumour aggressiveness.
- ▶ Cholangiocarcinoma shares a long-standing association with chronic inflammation and increased IL-6 expression.

What are the new findings?

- ▶ Progranulin expression is upregulated in cholangiocarcinoma via a mechanism involving IL-6-driven activation of ERK1/2 and subsequent RSK1 and C/EBP β activation.
- ▶ Progranulin exerts growth-promoting effects on cholangiocarcinoma via activation of Akt and nuclear extrusion of FOXO1.
- ▶ Inhibition of progranulin function slows cholangiocarcinoma growth.

How might it impact on clinical practice in the foreseeable future?

- ▶ Circulating progranulin concentration may prove useful as a prognostic marker of a number of tumour types.
- ▶ Targeting of progranulin may represent an alternative for the development of therapeutic strategies.

Figure 1.

Progranulin (PGRN) expression and secretion is increased in cholangiocarcinoma. PGRN levels were assessed in four cholangiocarcinoma cell lines as well as non-malignant cholangiocyte cell lines, H69 and HIBEC, by real time PCR and immunoblotting (A). For real time PCR, data are expressed as mean±SEM (n=4) (*p<0.05 compared with PGRN in H69 cells). Representative PGRN immunoblots are shown (lower panel). β-Actin is shown as a loading control. PGRN levels were also assessed in biopsy samples from 48 patients with cholangiocarcinoma and non-malignant controls by immunohistochemistry. Representative photomicrographs of the PGRN immunoreactivity are shown (B; magnification ×40). Staining intensity was assessed as described in the methods and expressed as mean±SEM for all patients with cholangiocarcinoma compared with control samples (B; *p<0.05 compared with PGRN immunoreactivity in control biopsy samples). PGRN levels in the supernatant of cell suspensions of cholangiocarcinoma cell lines and the non-malignant cholangiocyte cell lines, H69 and HIBEC, were determined by enzyme immunoassay (EIA) after 6 h (C). Data are expressed as mean±SEM PGRN concentration (ng/ml; n=3; *p<0.05 compared with PGRN levels secreted from H69 cells). PGRN levels in bile samples from cholangiocarcinoma and intrahepatic cholelithiasis patients was assayed by EIA (D). Data are expressed as mean±SEM PGRN concentration (ng/ml).

Figure 2.

Interleukin (IL)-6 regulates progranulin (PGRN) expression. Mz-ChA-1 cells were treated with recombinant IL-6 (10 ng/ml) for various time points up to 4 h (A). In parallel, stably transfected cell lines were established by transfecting Mz-ChA-1 cells with IL-6 shRNA vector. The resulting cell line (Mz-IL-6 shRNA) had only 4% of the IL-6 expression compared with the mock-transfected cell line (Mz-neo neg) (B). PGRN expression was assessed by real time PCR and immunoblotting in these treatment groups. Real time PCR data are expressed as mean $\pm$ SEM (n=4) (*p<0.05 compared with PGRN in control samples). Representative PGRN immunoblots are shown. β -Actin was used as the loading control.

Figure 3.

Interleukin (IL)-6 drives progranulin (PGRN) expression via a mechanism involving extracellular signal-regulated kinase (ERK)1/2 and ribosomal protein S6 kinase-1 (RSK1). Mz-ChA-1 cells were treated with rIL-6 (10 ng/ml) in the presence or absence of the ERK1/2 inhibitor, PD98059 (10 μM), or the RSK1 inhibitor, SL-0101-1 (0.2 μM). PGRN expression was assessed by real time PCR (A). ERK1/2 activation was assessed in Mz-ChA-1 cells after treatment with recombinant (r)IL-6 (10 ng/ml) for 1 h by immunoblotting using a phosphospecific ERK1/2 antibody (B). Data are expressed as relative phospho-ERK (pERK) levels after normalisation of the data to total ERK (tERK) levels in each sample. RSK1 activity was assessed in Mz-ChA-1 cells treated with rIL-6 (10 ng/ml) in the absence or presence of PD98059 (10 μM) for 1 h, by enzyme immunoassay activity kit (C). In parallel, basal ERK1/2 (D) and RSK1 (E) activity was assessed in Mz-IL-6 shRNA cells (lane 2) compared with the mock-transfected Mz-neo neg cell line (lane 1). All data are expressed as mean±SEM (n=3) (*p<0.05 compared with control samples).

Figure 4.

CCAAT-enhancer binding protein β (C/EBP β) is a downstream modulator of interleukin (IL)-6-driven progranulin (PGRN) expression. Mz-ChA-1 cells were treated with rIL-6 (10 ng/ml) in the presence or absence of the extracellular signal-regulated kinase (ERK)1/2 inhibitor PD98059 (10 μ M) or the ribosomal protein S6 kinase-1 (RSK1) inhibitor SL-0101-1 (0.2 μ M). C/EBP β activation was assessed by immunoblotting using a phosphospecific C/EBP β antibody (A). Data are expressed as relative phospho- C/EBP β (pC/EBP β) levels after normalisation of the data to total C/EBP β (tC/EBP β) levels in each sample. In parallel, basal C/EBP β activation was assessed in Mz-IL6 shRNA compared with Mz-Neo neg cells (B). The relative amount of C/EBP β bound to the PGRN promoter was assessed in Mz-IL-6 shRNA and Mz-Neo neg cells by chromatin immunoprecipitation (C) using a specific C/EBP β -specific antibody to precipitate the complex followed by real time PCR using specific primers for the PGRN promoter region. The effect of IL-6 on PGRN expression in cells with C/EBP β expression suppressed was determined. C/EBP β expression was knocked down in Mz-ChA-1 cells, and the resulting cell line (Mz-C/EBP β shRNA) was

treated with recombinant IL-6 (10 ng/ml) for 4 h. PGRN expression was assessed by real time PCR (D). All data are expressed as mean $\pm$ SEM (n=3; *p<0.05 compared with control samples).

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Figure 5.

Progranulin (PGRN) exerts proliferative effects on cholangiocarcinoma (A). Four cholangiocarcinoma cell lines were treated with various concentrations of recombinant (r)PGRN for 48 h. (B) Mz-ChA-1 cells were treated with rPGRN (1 µg/ml) in the absence or presence of the Akt inhibitor, FPA124 (1 µM). Cell proliferation was assessed using an MTS cell proliferation assay. Data are expressed as fold change in proliferation (mean±SEM; n=7; *p<0.05 compared with basal treatment within each cell line).

Figure 6.

Progranulin (PGRN) increases FOXO1 phosphorylation and nuclear extrusion. Mz-ChA-1 cells were treated with recombinant (r)PGRN (1 $\mu\text{g/ml}$) for various times up to 24 h. The levels of phospho FOXO1 and total FOXO1 were assessed by enzyme immunoassay-based activity kits (A). Data are expressed as fold increase of phospho FOXO1 (mean $\pm$ SEM; n=4; *p<0.05 compared with basal treatment). The subcellular location of FOXO1 was determined by immunofluorescence microscopy in Mz-ChA-1 cells treated with rPGRN (1 $\mu\text{g/ml}$) in the presence or absence of the Akt inhibitor, FPA124 (1 μM ; B). FOXO1 immunoreactivity is seen in red, and nuclei were counterstained with DAPI (blue). Scale bar=20 μm .

Figure 7.

Suppression of progranulin (PGRN) expression slows the rate of cholangiocarcinoma growth. PGRN expression was knocked down in Mz-ChA-1 cells, and the expression of proliferating cellular nuclear antigen (PCNA), as a marker of proliferative capacity, was assessed in the resulting cell line (Mz-PGRN shRNA), the parental cell line (Mz-ChA-1), and the mock-transfected control cell line (Mz-Neo neg) immunoblots (A). Data are expressed as mean±SEM (n=4) after normalisation to loading with β-actin (*p<0.05 compared with PCNA in Mz-ChA-1 cells). Representative PCNA immunoblots are shown. β-Actin is shown as a loading control. (B) Mz-ChA-1, Mz-Neo neg or Mz-PGRN shRNA cells were seeded into a 96-well plate and allowed to adhere overnight. Once adhered, the number of cells was counted in three non-overlapping fields per well and then again 24 h later. Data are expressed as fold increase in cell number per field (mean±SEM; *p<0.05). (C) In vivo, Mz-ChA-1 and Mz-PGRN shRNA cells were injected into the flank of athymic mice. After tumours were established (10 days), tumour volume was assessed, which was then considered to be 100%. Tumour volume was then assessed for a further 26 days, and the percentage increase in tumour volume determined (n=6).