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Targeting PDGFR- β in Cholangiocarcinoma

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Abstract

Background—Cholangiocarcinomas (CCAs) are highly desmoplastic neoplasms with a tumor microenvironment plentiful in myofibroblasts (MFBs). MFB-derived PDGF-BB survival signaling is a mediator of CCA cell resistance to apoptotic stimuli. This raises the concept that targeting PDGFR- β , a cognate receptor of PDGF-BB, represents a potential strategy for the treatment of human CCA.

Aims—Herein, we examine a role for inhibiting PDGFR- β in restoring CCA cell sensitivity to apoptotic stimuli *in vitro* and *in vivo*.

Methods—We employed human CCA samples from 41 patients (19 intrahepatic and 22 extrahepatic CCA samples), the human CCA cell lines KMCH-1 and HUCCT-1 as well as shPDGFR- β -KMCH-1 and human myofibroblastic LX-2 cells for these studies. *In vivo*-experiments were conducted using a syngeneic rat orthotopic CCA model.

Results—Of several MFB-derived growth factors profiled, PDGF-BB and CTGF were most abundantly expressed; however, only PDGF-BB attenuated TRAIL cytotoxicity. Co-culturing CCA cells with PDGF-BB-secreting MFBs significantly decreased TRAIL-induced CCA cell apoptosis as compared to monoculture conditions; this cytoprotective effect was abrogated in the presence of the tyrosine kinase inhibitors imatinib mesylate or linifanib, which inhibit PDGFR- β . Consistent with these findings, MFB-imparted cytoprotection also was abolished when PDGFR- β was knocked down as demonstrated in shPDGFR- β -KMCH-1 cells. Finally, administration of imatinib mesylate increased CCA cell apoptosis and reduced tumor growth in a rodent *in vivo*-CCA model that mimics the human disease.

Conclusions—Targeting PDGFR- β sensitizes CCA cells to apoptotic stimuli and appears to be therapeutic *in vivo*.

Keywords

hepatic stellate cells; imatinib mesylate/**STI-571**; linifanib/**ABT-869**; myofibroblasts; platelet-derived growth factor receptor beta

Cholangiocarcinoma (CCA) is the second most common primary hepatic cancer and its incidence in Western Countries is increasing (1). It is an extremely lethal malignancy with a

dismal overall prognosis and limited therapeutic options (2–4). Human CCAs *in vivo* paradoxically express and are resistant to the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (5–7). These observations suggest CCA tumor development and progression is maintained by potent survival signals. However, the mechanisms of CCA cell resistance to apoptotic stimuli are complex and further clarification is required in order to develop more effective therapies.

CCAs are highly desmoplastic neoplasms with a tumor microenvironment plentiful in myofibroblasts (MFBs). MFBs or cancer-associated fibroblasts display a permanently activated phenotype and characteristically express α -smooth muscle actin (α -SMA) (8). Within the liver, MFBs are largely derived from hepatic stellate cells (HSCs) as well as periportal fibroblasts (9, 10). There is an emerging role recognized for MFBs in tumor biology, such that cross-talk between MFBs and cancer cells appears to be exploited by many malignancies as a tumor-promoting mechanism (8, 11–13). Interestingly, in CCA the number of MFBs correlates with tumor size and patient survival (14, 15). MFBs also are capable of imparting strong survival signals as they were shown to decrease apoptosis of non-malignant cholangiocytes under co-culture conditions (16). However, the mechanisms of the tumorigenic MFB:CCA cell cross-talk is incompletely understood.

Platelet-derived growth factors (PDGFs) are known to be important mediators of cholangiocyte:fibroblast paracrine cross talk in rodent models of biliary tract inflammation and fibrogenesis (16, 17). Five PDGF isoforms have been described including PDGF-AA, -BB, -AB, CC and DD (18). In MFBs, PDGF-BB appears to be the isoform predominantly expressed (19). There are two cognate receptors, platelet-derived growth factor receptor (PDGFR)- α and - β (18) and PDGFR- β (the receptor for PDGF-BB) is expressed by CCA cells (20). PDGFR- β can be blocked by tyrosine kinase inhibitors such as imatinib mesylate/**STI-571** or linifanib/**ABT-869** (18, 21, 22). We have recently reported that MFB-derived cell PDGF-BB imparts survival signaling in CCA cells by co-activation of the Hedgehog (Hh) signaling pathway (20). In these studies, the Hh inhibitor cyclopamine was therapeutic as it increased the susceptibility of CCA cells to TRAIL cytotoxicity (20). However, whether targeting PDGFR- β directly is also therapeutic was not explored.

The objective of this study was to examine whether direct targeting of PDGFR- β by tyrosine kinase inhibitors would result in a sensitization of CCA cells to TRAIL-induced apoptosis. The results suggest that blocking PDGFR- β survival signaling overcomes CCA cell resistance to TRAIL cytotoxicity *in vitro* and *in vivo*. These observations have implications for the treatment of human CCA.

MATERIALS AND METHODS

Materials

rhTRAIL (R&D Systems, Minneapolis, MN) and rhCTGF (PeproTech Rocky Hill, NJ) were prepared according to the suppliers protocols. Imatinib mesylate/**STI-571**, an inhibitor of the kinase activity of the PDGFR(- β), was a generous gift from E. B. Leof (Div. of Pulmonary and Critical Care Medicine, Mayo Clinic, Rochester, MN). Imatinib mesylate was dissolved in sterile water (10 mmol/L stock solution) and subsequently diluted in cell culture medium. Linifanib/**ABT-869**, another inhibitor of the kinase activity of the PDGFR(- β), was purchased from Selleck (Houston, TX), dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO; 10 mmol/L stock solution) and also subsequently diluted in cell culture medium for use in *in vitro* experiments.

Cell lines/culture and human samples

The human CCA cell lines KMCH-1, HUCCT-1, and Mz-CHA-1, the erythroblastic leukemia viral oncogene homolog (ErbB-2)/neu transformed malignant rat cholangiocyte cell line BDeneu (*in vivo* experiment) as well as the LX-2 cells, an immortalized myofibroblast cell line derived from human HSCs, were cultured as previously described (20, 23–26). Similar conditions were used in the co-culture experiments. Intrahepatic (n = 19) and extrahepatic (n = 22) CCA samples from 41 patients were collected with Institutional Review Board approval.

Generation of a stable transfectant expressing PDGFR- β short hairpin RNA

Short hairpin RNA (shRNA) lentiviral plasmid for PDGFR- β was obtained from Thermo Fisher Scientific/Open Biosystems (Huntsville, AL; Oligo ID: V2LHS_169803; GenBank accession no.: NM_002609). KMCH-1 cells were transfected using OptiMEM I (Gibco-Invitrogen, Carlsbad, CA) containing 6 μ L/mL Lipofectamine (Invitrogen), 1 μ g/mL plasmid DNA, and 6 μ L/mL Plus reagent (Invitrogen). Forty-eight hours after transfection, fresh DMEM containing 0.5 μ g/mL puromycin was added. Surviving clones were separated using cloning rings and individually cultured. A clone with a scrambled shRNA was employed as a control (stable scrambled KMCH-1 cells). The expression/knockdown of PDGFR- β in the clones was assessed by immunoblot analysis.

Real-time polymerase chain reaction

Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) and was reverse-transcribed with Moloney leukemia virus reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). Quantitation of the complementary DNA template was performed with real-time polymerase chain reaction (PCR; LightCycler, Roche, Indianapolis, IN) using SYBR green (Roche) as a fluorophore (27). Oligonucleotide sequences and expected product sizes for all primer pairs used for quantitative RT-PCR analysis are shown in Table 1. As an internal control, primers for 18S rRNA (Ambion, Austin, TX) were employed. Using gel purified amplicons, a standard curve was generated to calculate the copy number/ μ L. The target mRNA expression of each sample was calculated as the copy ratio of target mRNA to 18S rRNA and then normalized to the target mRNA expression of PDGF-B or vehicle, respectively.

Co-culture experiments

Cell co-culture experiments were performed using a transwell insert co-culture system (24 wells) equipped with 0.4 μ m pore size polyester (PET) inserts (Corning Costar, Acton, MA) for 2 days according to the manufacturer's recommendations. Briefly, KMCH-1 or shPDGFR- β -KMCH-1 cells were plated alone or together with LX-2 cells in the transwell insert co-culture system (KMCH-1 or shPDGFR- β -KMCH-1 in the bottom wells and LX-2 cells in the inserts; 1:1 ratio). First, all cells were plated alone overnight. The co-culture insert chambers with the LX-2 cells were then transferred the next day. Cells were treated as indicated and rhTRAIL was added at the end of the experiment (day 2) for 6 hrs whereas imatinib mesylate or linifanib was added for 24 hrs (day 2). After rhTRAIL treatment, the KMCH-1 cells (normal, stable scrambled, or shPDGFR- β KMCH-1) in the bottom wells were analyzed for apoptosis by DAPI-staining/TUNEL assay as described in the "Quantitation of apoptosis" section (for the TUNEL assay, cells were plated on sterilized trimmed coverslips that were placed in the bottom wells prior to cell seeding).

Quantitation of apoptosis

Apoptosis in CCA cells was quantified by assessing the characteristic nuclear changes of apoptosis after staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma,

St. Louis, MO) using fluorescence microscopy (28). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays (cell co-culture and rat CCA samples) were carried out using the In situ Cell Death Detection kit (Roche, Indianapolis, IN) according to the supplier's protocol and as previously described (20).

Immunohistochemistry for PDGFR- β

Immunohistochemistry was performed using formalin-fixed, paraffin-embedded human CCA samples. Slides were deparaffinized in xylene and rehydrated through sequential graded ethanol steps. The antigen retrieval was performed by permeabilizing the slides in 0.1% Triton X 100 for 2 min and incubation in sodium citrate (0.01 M sodium citrate, 0.05% Tween 20; pH 6.0) for 30 min using a vegetable steamer. After cooling, further steps were carried out according to the protocols of the EnVision+ System-HRP [DAB] detection kit (K4010 [anti-rabbit]; Dako, Carpinteria, CA). The primary antiserum against PDGFR- β (1:25; Santa Cruz, Santa Cruz, CA; P 20) was applied overnight at 4°C. Finally, the slides were counterstained with Mayer's Hematoxylin Solution (Sigma, St. Louis, MO), mounted and examined by light microscopy.

Immunoblot analysis

Whole cell lysates were obtained as previously described (29). Primary antisera used were: Actin (1:2000; Santa Cruz, Santa Cruz, CA; C-11) and PDGFR- β (1:1000; Santa Cruz, Santa Cruz, CA; P-20). Horseradish peroxidase-conjugated secondary antibodies for rabbit (Santa Cruz; sc-2004) and goat (Santa Cruz; sc-2020) were incubated at a dilution of 1:3000 for 1 hr at RT. Proteins were visualized using enhanced chemiluminescence reagents (ECL, Amersham Biosciences, Buckinghamshire, UK) and Kodak X-OMAT films.

Immunofluorescence microscopy for c-kit and cytokeratin 7

Immunohistochemistry was performed using formalin-fixed, paraffin-embedded rat CCA samples. Slides were deparaffinized in xylene and rehydrated through sequential graded ethanol steps. For c-kit- and cytokeratin 7 (CK7)-co-staining, the antigen retrieval was performed by permeabilizing the slides in 0.1% Triton X 100 for 2 min and incubation in deionized water containing 5% urea using a vegetable steamer for 20 min. (for CK7/TUNEL co-staining, an additional antigen retrieval step was performed with sodium citrate followed directly by cooling and application of the TUNEL reaction mix; the TUNEL assay is described in the "Quantitation of apoptosis" section) The primary antisera/antibodies against c-kit (1:100; Santa Cruz, Santa Cruz, CA; M-14) and CK7 (1:10; Abcam; ab9021) were applied overnight at 4 °C. After washing, the slides were incubated with Alexa Fluor® 488 rabbit anti-goat IgG (c-kit; 1:1000; Invitrogen, Camarillo, CA; A11078) and then Texas Red®-X goat anti-mouse IgG (CK7; 1:1000; Invitrogen; T6390) for 1 hr in the dark at RT. The slides were then washed three times in PBS, one time in water and mounted using Prolong Antifade with DAPI (Invitrogen). The slides were analyzed by fluorescent confocal microscopy (LSM 510; Zeiss, Jena, Germany).

Animal experiments

All animal studies were performed in accordance with and approved by the Institutional Animal Care and Use Committee. *In vivo* intrahepatic cell implantation (syngeneic rat orthotopic CCA model) was carried out in male adult Fischer 344 rats (Harlan, Indianapolis, IN) with initial body weights between 195 and 230 g as previously described (20, 24, 26). Imatinib mesylate (30 mg/kg BW; approx. 0.5 mL) or vehicle (normal saline) was given intraperitoneally every day for one week (1st injection: 7th post-operative day; 7th injection: 13th post-operative day). Twenty-four hours after receiving the last injection, the rats were euthanized and the livers removed for further analysis. To assess the number of metastases-

free and metastases-bearing rats, the abdominal cavities, the retroperitoneal spaces and the thoracic cavities were thoroughly examined as previously described (20, 24).

Statistical analysis

Data are expressed as the mean $\pm$ s.e.m. and represent at least 3 independent experiments. Differences in experiments with two groups were compared using the two-tailed Student *t*-test or the χ^2 test for discrete data. Differences in experiments with more than two groups were compared using ANOVA with Bonferroni post hoc correction. Differences were considered as significant at levels of $p < 0.05$.

RESULTS

PDGF-B is a prominently expressed myofibroblast-derived survival factor

Initially, we compared the mRNA expression of PDGF-B with other MFB-derived, CCA-relevant growth factors (PDGF-A, fibroblast growth factors [FGFs] 5 and 10 as well as connective tissue growth factor [CTGF]) in the human MFB cell line LX-2 (Fig. 1A). Among the growth factors profiled, PDGF-B and CTGF displayed the most abundant mRNA expression levels. In contrast to PDGF-BB, CTGF did not impart significant survival signals for CCA cells (KMCH-1 and HUCCT-1) *in vitro* (Fig. 1B and C). Thus, MFB cells not only secrete higher amounts PDGF-BB than several CCA cell lines (20), but mRNA of PDGF-B is also expressed at high levels in comparison with other MFB-derived growth factors. Unlike CTGF, PDGF-BB additionally attenuates TRAIL cytotoxicity (Fig. 1B and C) (20) and, therefore, can be considered a survival factor.

Targeting PDGFR- β promotes CCA cell apoptosis

Initially, we examined the expression of PDGFR- β in CCA samples of 41 patients (19 intrahepatic and 22 extrahepatic CCA samples) by immunohistochemistry. PDGFR- β -immunoreactive CCA cells were present in 89 % (17 out of 19) of the intrahepatic and 68 % (15 out of 22) of the extrahepatic CCA samples (Fig 2A). Given the role of PDGF-BB as a survival factor, we next determined if targeting its receptor PDGFR- β restores CCA cell sensitivity to TRAIL killing. Therefore, we examined the effect of co-culturing KMCH-1 cells (which express PDGFR- β) (20) with PDGF-BB-secreting LX-2 cells on TRAIL-induced CCA cell apoptosis in the presence or absence of imatinib mesylate (Fig. 2B and C) (21). As measured by either nuclear morphology (Fig. 2B) or TUNEL assay (Fig. 2C), KMCH-1 cells were more resistant to TRAIL-induced apoptosis when co-cultured with LX-2 cells as compared to monoculture conditions, a cytoprotective effect that was significantly attenuated by imatinib mesylate (Fig. 2B and C). A similar inhibitory effect on MFB-imparted cytoprotection was observed by linifanib (Fig. 2B and C), another receptor tyrosine kinase inhibitor which potently blocks PDGFR- β (22). As imatinib mesylate also blocks tyrosine-protein kinase kit (c-kit) signaling (21, 30, 31), we assessed mRNA expression of c-kit (and PDGFR- β) in KMCH-1 cells by RT-PCR analysis (Fig. 3A). KMCH-1 cells expressed PDGFR- β but not c-kit. Thus, the promotion of KMCH-1 cell apoptosis by imatinib mesylate observed in the co-culture studies is unlikely mediated via inhibition of c-kit. In further experiments, we employed shPDGFR- β -KMCH-1 cells to exclude the possibility that these inhibitors were effecting survival pathways by blocking receptor tyrosine kinases other than PDGFR- β (Fig 3C); PDGFR- β knockdown was confirmed by immunoblot analysis (Fig. 3B). Consistent with the previous observation (Fig. 2B and C), PDGFR- β knockdown precluded the survival effect of the LX-2 cell co-culture paradigm (Fig. 3C). Thus, PDGFR- β is widely expressed in human CCA cell lines (20) as well as CCA tissue; moreover, targeting PDGFR- β in CCA cells abrogates the survival advantage afforded these cancer cells against TRAIL cytotoxicity when cultured in the presence of MFBs.

Imatinib promotes CCA cell apoptosis and is tumor suppressive *in vivo*

To determine if the proapoptotic *in vitro*-effect of PDGFR- β signaling inhibition by imatinib mesylate observed in co-cultures is translatable to an *in vivo* model, we employed a syngeneic rat orthotopic CCA model (BDeneu malignant cells injected into the liver of male Fischer 344 rats) (24, 26). This preclinical rodent model of CCA duplicates several characteristic features observed in human CCA tissue. For example, BDeneu cells also express TRAIL as well as PDGFR- β *in vivo*, and PDGF-BB expression is apparent in α -SMA-positive MFBs within a desmoplastic tumor stroma (20, 24, 32). In order to complete the profiling of study-relevant proteins in this *in vivo* CCA model, we performed immunohistochemistry for c-kit (Fig. 4A) and found c-kit to be expressed in the tumorous glands. Prior to the apoptosis studies, we also investigated whether imatinib mesylate had a confounding *in vivo*-effect on MFB growth within the tumor stroma (known to be linked to PDGFR signaling) by assessing mRNA expression of the MFB marker α -SMA via quantitative RT-PCR (8). The α -SMA mRNA expression levels were not significantly different in CCA specimens of imatinib mesylate-treated rats as compared to controls (Fig. 4B); an observation excluding a significant effect of imatinib on MFBs. In contrast, CCA cell apoptosis was increased in animals treated with imatinib mesylate as compared to vehicle-treated rats (Fig. C). CCA cell apoptosis was confirmed by demonstrating colocalization of TUNEL-positive cell nests with tumorous glands displaying CK7 (a biliary epithelial cell marker expressed by CCA cells, but not MFBs; Fig. 4C middle). Thus, imatinib mesylate promotes apoptosis of CCA cells but not MFBs in an *in vivo* rodent model of CCA.

Consistent with the proapoptotic effects of imatinib mesylate in the syngeneic rat orthotopic CCA model (BDeneu malignant cells injected into the liver of male Fischer 344 rats) (24, 26), imatinib mesylate was also effective in reducing tumor size and metastasis (Fig. 5). Indeed, tumor weight, and tumor/liver as well as tumor/body weight ratios were significantly decreased in imatinib mesylate-treated rats (Fig. 5 A–C and E). In addition, 100% of the rats treated with imatinib mesylate displayed no extrahepatic metastases, whereas only 57% of the vehicle-treated animals were free of metastases (three out of seven animals in this group showed tumors predominantly occurring in the greater omentum and peritoneum; Fig. 5D). Taken together, these data suggest that imatinib mesylate decreases tumor growth as well as metastasis in an *in vivo* rodent model of CCA.

DISCUSSION

The results of this study provide new insights regarding targeting cytoprotective MFB-to-tumor cell paracrine signaling as a therapeutic approach for the treatment of human CCA. These data indicate that: (i) PDGF-BB and CTGF are abundantly expressed by MFBs but that PDGF-BB is a more potent survival factor than CTGF; (ii) targeting PDGFR- β survival signaling sensitizes CCA cells to TRAIL-induced cell death *in vitro*; and (iii) imatinib mesylate promotes CCA cell apoptosis resulting in tumor suppression in an orthotopic syngeneic rodent *in vivo* CCA model. These findings are illustrated in Fig. 6 and discussed in greater detail below.

We recently reported that MFB-to-cancer cell PDGF-BB paracrine signaling imparts survival signals for CCA (20). Interestingly, PDGF-BB survival signaling in the prior study was mediated by co-activation of the Hedgehog (Hh) signaling pathway. Indeed, Hh signaling inhibition by cyclopamine increased the susceptibility of CCA cells to apoptotic stimuli (20). PDGF-BB by activating protein kinase A increased trafficking of Hh signaling mediator smoothened to the plasma membrane thereby activating Hh signaling pathways. Thus, PDGFR- β activation was upstream of smoothened activity suggesting that PDGFR- β inhibition would preclude activation of this collective survival pathway. Therefore, we

explored whether more direct targeting PDGFR- β signaling would be similarly effective in blocking CCA survival signaling. In co-culture experiments, MFB cytoprotection against TRAIL-induced apoptosis was abrogated when PDGFR- β signaling was blocked by imatinib mesylate or linifanib, another receptor tyrosine kinase inhibitor which potently blocks PDGFR- β and is currently being tested in clinical phase II/III studies for the treatment of human hepatocellular carcinoma (22). Similar results were obtained by stably knocking down PDGFR- β in CCA cells. These observations suggest PDGFR- β survival signaling represents a potential target for the treatment of human CCA.

The effect of imatinib mesylate as a single-agent or in combination with chemotherapeutics on CCA cell viability/apoptosis has also been investigated by other groups (30, 31). In contrast to our findings, imatinib mesylate in these studies was less effective in CCA cell lines not expressing the c-kit receptor. However, these studies did not investigate cytoprotection against TRAIL-induced apoptosis which is a characteristic feature of CCA as these cancers paradoxically express and are resistant to TRAIL (5–7). Additionally, these experiments were performed under monoculture conditions and not designed to examine functional interactions between MFBs and tumor cells in which signaling via PDGFR- β appears to play a predominant role.

The orthotopic, syngeneic rodent CCA model used in the present study recapitulates the molecular signature and TRAIL expression of human CCA (24, 26). It also provides a syngeneic, non-immunocompromised tumor microenvironment rich in activated α -SMA-secreting MFBs and mimics the cellular expression patterns of PDGF-BB and PDGFR- β found in the human disease (20). Kamenz *et al.* also reported that imatinib mesylate suppresses CCA tumor volume and mass *in vivo* (in a xenograft mouse CCA model) (30). Herein, we extended these prior observations by demonstrating a functional interaction between the tumor microenvironment and CCA cells. Moreover, we demonstrate that PDGFR- β signaling inhibition increases apoptosis of CCA cells *in vivo*. Similar to the study of Kamenz *et al.* (30), the rodent model of CCA employed in this study expresses the c-kit receptor *in vivo*. Our *in vitro* observations are most consistent with the tumor suppressive *in vivo*-effects of imatinib mesylate being mediated by MFB-to-CCA cell PDGF-BB signaling inhibition; however, we cannot exclude a contribution of c-kit inhibition to the reduction of CCA tumor size in the imatinib mesylate group.

Imatinib mesylate and sorafenib (a multikinase inhibitor also capable of PDGFR inhibition) are currently being tested for the treatment of human CCA in phase II clinical trials (33). The first published results suggest imatinib mesylate to be efficacious as first line treatment of CCA (33). However, these findings are preliminary and larger controlled clinical studies including more selective PDGFR- β inhibitors (as single-agents or in combination with chemotherapeutics) are needed to evaluate the use of PDGFR- β signaling inhibition for the treatment of human CCA. In conclusion, direct targeting of CCA PDGFR- β survival signaling appears to sensitize CCA cells to TRAIL-induced apoptosis. These observations support the development of more selective PDGFR- β inhibitors for the treatment of human CCA.

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Figure 1.

PDGF-B is an abundantly expressed myofibroblast-derived survival factor. (A) MFBs express PDGF-B mRNA growth factors. Quantitative RT-PCR analysis for mRNA expression of the platelet-derived growth factors (PDGFs) -A and -B, the fibroblast growth factors (FGFs) 5 and 10 as well as connective tissue growth factor (CTGF) was performed in the human myofibroblast (MFB) cell line LX-2. The target mRNA expression of each sample is calculated as the copy ratio of target mRNA to 18S rRNA and then normalized to the target mRNA expression of PDGF-B (n=3). (B and C) First, KMCH 1 (B) or HUCCT-1 (C) cells (either serum-starved for 2 days) were treated with vehicle, PDGF BB (200 ng/mL), or CTGF (200 ng/mL) for 8 hrs. PDGF-BB and CTGF were retained in the culture media, and the cells were then treated as indicated with rhTRAIL (5 ng/mL) for additional 6 hrs. Apoptosis was measured by DAPI staining with quantitation of apoptotic nuclei by fluorescence microscopy. Mean $\pm$ s.e.m., n=3.

Figure 2.

Targeting PDGFR- β signaling with tyrosine kinase inhibitors promotes TRAIL cytotoxicity in CCA cells. (A) PDGFR- β expression in 19 intrahepatic and 22 extrahepatic human CCA samples was examined by immunohistochemistry. (B and C) KMCH-1 cells were plated alone (monoculture) or together with PDGF-BB-secreting LX-2 cells (co-culture) in a transwell insert co-culture system (KMCH-1 cells in the bottom wells and LX-2 cells in the inserts; 1:1 ratio) for 2 days. Cells were treated as indicated with vehicle, rhTRAIL (10ng/mL for 6 hrs on day 2), rhTRAIL plus imatinib (rhTRAIL: 10ng/mL for 6 hrs on day 2; Imatinib: 5 μ mol/L for 24 hrs [day2]), or rhTRAIL plus linifanib (rhTRAIL: 10ng/mL for 6 hrs on day 2; Linifanib: 0.5 μ mol/L for 24 hrs [day2]). After rhTRAIL treatment for 6 hrs, KMCH-1 cells were analyzed for apoptotic nuclear morphology by DAPI-staining (B) and

for DNA fragmentation by TUNEL-assay (C) with quantitation of apoptotic nuclei by fluorescence microscopy. Mean $\pm$ s.e.m., n=5.

Figure 3.

Targeting PDGFR- β signaling genetically promotes TRAIL cytotoxicity in CCA cells. (A) mRNA expression of the imatinib target receptors PDGFR- β and tyrosine-protein kinase kit (c kit) was assessed in KMCH-1 cells by qualitative RT PCR analysis. A 100bp DNA ladder is shown in the first lane. mRNA from Mz-CHA-1 cells which have been reported to express c-kit (31) served as positive control (forth lane). (B) PDGFR- β expression/knockdown in scrambled and shPDGFR- β -KMCH-1 cells was assessed by immunoblot analysis. (C) stable scrambled or shPDGFR- β -KMCH-1 cells were plated alone (monoculture) or together with PDGF-BB-secreting LX-2 cells (co-culture) in a transwell insert co-culture system (stable scrambled or shPDGFR- β -KMCH-1 cells in the bottom wells and LX-2 cells in the inserts; 1:1 ratio) for 2 days. Cells were treated as indicated with vehicle or rhTRAIL (10ng/mL for 6 hrs on day 2). After rhTRAIL treatment for 6 hrs, stable scrambled or shPDGFR- β -KMCH-1 cells were analyzed for apoptotic nuclear morphology by DAPI-staining with quantitation of apoptotic nuclei by fluorescence microscopy. Mean $\pm$ s.e.m., (n=5).

Figure 4.

Imatinib administration promotes apoptosis in CCA cells *in vivo*. A syngeneic rat orthotopic model of CCA (BD Eneu cells; Fischer 344 rats) was employed for this examination. (A) c-kit expression in tumor tissues of untreated rats (14 days after tumor cell implantation into the left lateral liver lobe) was examined by immunohistochemistry. c-kit expression (green) colocalizes to CCA glands (dotted lines) also expressing cytokeratin 7 (CK7, a CCA cell marker; red) resulting in a yellow c-kit signal (green-red overlay). Nuclei are counterstained with DAPI (blue). (B) CCA specimens of imatinib (30 mg/kg BW intraperitoneally daily for one week; 1st injection: 7th post operative day, 7th injection: 13th post operative day)- or vehicle-treated tumor-bearing rats were analyzed for mRNA expression of the MFB marker α -SMA by quantitative RT-PCR. Mean $\pm$ s.e.m., n=3. (C) Apoptotic nuclei were assessed in tumor tissues by TUNEL staining (green) and the identity of TUNEL-positive cells confirmed by co-staining via immunohistochemistry for CCA cell marker CK7 (red; CCA cell glands within the tumor stroma are illustrated by white dotted lines). Nuclei are counterstained with DAPI (blue). Animals were treated with vehicle (left) or imatinib (middle). Quantitation of TUNEL-positive cells (expressed as number per high power field [HPF]) demonstrates that in imatinib-treated animals CCA cell apoptosis was increased as compared to controls (right; Mean $\pm$ s.e.m., n=10).

Figure 5.

Imatinib administration reduces tumor growth and metastasis *in vivo*. A syngeneic rat orthotopic model of CCA (BDenu cells; Fischer 344 rats) was employed for this examination. In imatinib (30 mg/kg BW intraperitoneally daily for one week; 1st injection: 7th post-operative day, 7th injection: 13th post-operative day)- or vehicle-treated tumor-bearing rats tumor/liver/body weight and extrahepatic metastasis were assessed 14 days after tumor cell implantation into the left lateral liver lobe. (A–C) Changes in tumor weight (A) as well as tumor/liver (B) and tumor/body (C) weight ratios are depicted as bar graphs. Mean $\pm$ s.e.m., $n=7$. (D) The stacked column plot indicates the numbers of animals with and without metastases for vehicle- and imatinib-treated groups ($p = 0.051$ by χ^2 test). (E) Depicted are representative explanted livers of vehicle- (left) and imatinib-treated (right) rats (arrows indicate the liver tumors).

Figure 6.

Schematic diagram illustrating the role of targeting PDGFR- β in sensitizing CCA cells to TRAIL-induced apoptosis. (A) PDGF-BB, mainly secreted by myofibroblasts, via PDGFR- β promotes resistance against cytotoxicity by endogenous TRAIL. (B) Tyrosine kinase inhibitors such as imatinib mesylate or linifanib block PDGFR- β survival signaling and, thus, promote apoptosis in CCA cells.

Table 1

Primer sequences and expected product sizes of human and rat primer pairs used for quantitative RT-PCR analysis

Gene	Primer sequence	Product length (reference)
PDGF-A	forward 5'-CCCCTGCCCATTTCGGAGGAAGAG-3'	227 bp (34)
	reverse 5'-TTGGCCACCTTGACGCTGCGGTG-3'	
PDGF-B	forward 5'-GATCCGCTCCTTTGATGATC-3'	435 bp (34)
	reverse 5'-GTCTCACACTTGCATGCCAG-3'	
FGF5	forward 5'-TGCAAGTGCCAAGTTCACAGA-3'	91 bp
	reverse 5'-AGTTCTATGTATTGCTGAGGCATAGGTA-3'	
FGF10	forward 5'-GCGGGAAGGCATGTGCGGAG-3'	151 bp
	reverse 5'-TCTCCAGGATGCTGTACGGGC-3'	
CTGF	forward 5'-AGTGGGTGTGTGACGAGCCAA-3'	494 bp (35)
	reverse 5'-GTAATGGCAGGCACAGGTCTTG-3'	
PDGFR- β	forward 5'-AATGTCTCCAGCACCTTCGT-3'	688 bp (34)
	reverse 5'-AGCGGATGTGGTAAGGCATA-3'	
c-kit	forward 5'-AAGGACTTGAGGTTTATTCCT-3'	345 bp (36)
	reverse 5'-CTGACGTTTCATAATTGAAGTC-3'	
α -SMA *	forward 5'-TGTGTGAAGAGGAAGACAGCAC-3'	462 bp (37)
	reverse 5'-GCACAATACCAGTTGTACGTCC-3'	

All primers were designed to have an optimum annealing temperature between 51 and 59 °C.

* rat primer pair.