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Download date: 17 Oct 2017
Chapter 3

Protease-activated receptor 2 suppresses lymphangiogenesis and subsequent lymph node metastasis in a murine pancreatic cancer model.

Kun Shi, Karla CS Queiroz, Joris JTH Roelofs, Carel JM van Noesel, Dirk J Richel and C Arnold Spek

Abstract

Protease-activated receptor-2 (PAR-2) is a G protein-coupled receptor that functions as a cell-surface sensor for coagulation factors and other proteases associated with the tumour microenvironment. Pancreatic cancer cells express high levels of PAR-2 and activation of PAR-2 may induce their proliferation and migration. Interestingly, however, PAR-2 expression is increased in stroma-rich pancreatic cancer regions, suggesting a potential role of PAR-2 in the tumour microenvironment. Here, we assessed the importance of PAR-2 in the stromal compartment by utilizing an orthotopic pancreatic cancer model, in which tumour cells are PAR-2-positive, whereas stromal cells are PAR-2-negative. We assessed tumour weight and volume and analysed proliferation and (lymph)angiogenesis both in vivo and in vitro. We show that genetic ablation of PAR-2 from the stromal compartment inhibits primary tumour growth, which is accompanied by reduced vascularization in primary tumours and reduced in tube formation of vascular endothelial cells in vitro. In contrast to smaller primary tumours, the number of lymph node metastases was increased in PAR-2-deficient animals, which was accompanied by an increased number of lymphatic vessels. In vitro tube-formation assays show that PAR-2 does not inhibit the intrinsic tube-forming capacity of lymphatic endothelial cells, but that PAR-2 actually inhibits cancer cell-induced tube formation. Overall, stromal PAR-2 thus plays a dual role in pancreatic cancer development by potentiating primary tumour growth but limiting lymphangiogenesis and subsequent lymph node metastasis. Our data identify a novel role of PAR-2 in the tumour microenvironment and pinpoint PAR-2 as a negative regulator of lymphangiogenesis.
Introduction

Pancreatic ductal adenocarcinoma is one of the most lethal solid malignancies, which is associated with a high propensity for local invasion and distant metastases [1]. Although the treatment of cancer in general has improved significantly over recent decades, the overall 5 year survival rate of pancreatic cancer is still<5% [2] and overall mortality rates approach 99% [3]. The high mortality results from the majority of patients presenting with locally advanced and/or metastatic disease, which is rapidly progressive and inevitably fatal. In<20% of pancreatic cancer patients the tumour is surgically removed, which leads to improved 5 year survival rates of around 15–20%. Nevertheless, the majority of these patients eventually also succumb to metastatic disease [4].

Pancreatic cancer is among the most common malignancies associated with thromboembolic events [5] and these events confer a significantly worse prognosis [6]. Interestingly, the hypercoagulable state in pancreatic cancer patients not only acts as an important risk factor for thrombosis but may actually play a role in tumour progression and metastasis [7,8]. Indeed, anticoagulant treatment significantly improved survival in patients with locally advanced or metastatic pancreatic carcinoma [9], although this beneficial effect of low molecular weight heparin could not be recapitulated in a recent randomized trial [10].

Protease-activated receptor-2 (PAR-2) is a seven-transmembrane domain receptor belonging to the family of G protein-coupled receptors [11]. Proteolytic removal of the N-terminal extracellular domain by (amongst others) the tissue factor (F) VIIa complex and by coagulation FXa [12] unMASKS a novel tethered ligand that interacts with the body of the receptor [13]. Subsequent transmembrane signalling triggers a variety of signaling pathways affecting diverse pathophysiological responses. In the setting of pancreatic cancer, PAR-2 may stimulate the proliferation of cancer cells [14,15], although we recently did not observe any effect of PAR-2
stimulation on pancreatic cancer cell proliferation [16]. Moreover, PAR-2 stimulates pancreatic cancer cell migration [16] and does induce interleukin-8 release [17]. Finally, intratumoral treatment of Panc1 xenografts with siRNA targeting PAR-2 suppresses tumour growth in nude mice [18]. Overall, these data suggest that PAR-2 contributes to tumour progression and is over-expressed in malignant versus non-malignant human pancreatic tissue sections [19]. Interestingly, PAR-2 expression is significantly increased in stroma-rich tumour regions, suggesting a potential role of PAR-2 in the stromal compartment of pancreas cancer.

Here we specifically addressed the importance of PAR-2 expression in the pancreatic tumour stroma by evaluating tumour growth and metastasis in an orthotopic pancreatic cancer model, in which the tumour cells are PAR-2-positive whereas stromal cells are PAR-2-negative.

**Materials and methods**

**Patient tissue microarray**

Tumour sections of pancreatic cancer patients obtained during surgery were used according to Dutch law and the guidelines of the Medical Ethical Committee of the Academic Medical Centre (Amsterdam, The Netherlands). According to these guidelines, tumour sections can be freely used, without informed consent, after anonymization.

**Animals**

Wild-type, 8 week-old, C57Bl/6 mice were purchased from Charles River (Someren, The Netherlands). Homozygous PAR-2-deficient C57Bl/6 mice were originally provided by Jackson Laboratories (ME, USA) and bred at the animal care facility of the Academic Medical Centre. Wild-type and PAR-2-deficient mice had similar body weight and growth characteristics.
throughout the experiments. All mice were maintained according to institutional guidelines. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. The Animal Care and Use Committee of the Academic Medical Centre approved all experiments.

**Orthotopic pancreatic cancer model**

Mice were subjected to an orthotopic model of pancreatic cancer, essentially as described previously [20,21]. Briefly, confluent cultures of PANC02 cells (>90% viable; kindly provided by Dr Schmitz, Universitäts Klinikum Bonn) were detached by TrypLE™ Select (Invitrogen) and centrifuged at 800 rpm for 5 min, washed twice in phosphate-buffered saline (PBS) and resuspended in 0.9% sterile saline (Sigma, St Louis, MO, USA). Tumour cells (4 × 10^5 cells/mouse) were injected directly into the pancreas. The mice were evaluated daily for changes in body weight and signs of discomfort or morbidity and were euthanized 5 weeks after tumour cell injection. The pancreas, including the tumour, was removed, measured, weighed and preserved in formalin. Lymph nodes and distant organs were also removed and fixed in formalin.

**Immunohistochemistry**

Histological examination was performed essentially as described previously [22]. Briefly, excised tumours were fixed in formalin, embedded in paraffin and 4 μm thick sections were subsequently deparaffinized, rehydrated and washed in deionized water. The sections were stained with haematoxylin and eosin (H&E) according to routine procedures. For immunohistochemistry of PAR-2, Ki67, CD31 and D2-40, endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide for 15 min at room temperature, and antigen retrieval was performed for 10 min at 96 °C in 10 mM sodium citrate
buffer, pH6. Next, the sections were blocked for 10min with Ultra V block (Thermo Scientific, UK). Primary antibodies against PAR-2 (1:80 for human tissue and 1:200 for mouse tissue; SAM11, Santa Cruz Biotechnology) or Ki67 (1:500, clone Sp6; Neomarkers, USA) were added for overnight incubation at 4 °C, whereas the anti-CD31 antibody (1:1000; sc-1506-R, Santa Cruz Biotechnology, for mouse tissue, and 1:40, M0823, Dako, for human tissue sections) and anti-D2-40 antibody (1:200, M3619, Dako) were incubated for 90min at room temperature. The sections were subsequently incubated with appropriate HRP-conjugated secondary antibodies and DAB staining was used to visualize peroxidase activity. Lymph endothelial vessels were stained using an anti-LYVE-1 antibody (1:250; C225-3, MBL International, USA), followed by the UltraTech HRP Streptavidin–Biotin Detection System (PN IM2391, Beckman Coulter, The Netherlands). The sections were photographed using a microscope equipped with a digital camera (Leica CTR500, Leica Microsystems, Germany). CD31- and LYVE-1-positive vessels were counted in 10 different fields/slide at × 400 magnification. Ki67 stainings were analysed using ImageJ and expressed as percentage of surface area. Depending on tumour size, an average of 20–40 images/tumour at × 200 magnification was used for analysis.

Reagents

Human PAR-2 agonist peptide (hPAR-2-AP; H–Ser–Leu–Ile–Gly–Lys–Val–NH2, HPLC purity>99%, mass spec-verified) and PAR-2 antagonist P2pal-18S(palmitate-RSSAMIDENEKRRKSKAIK–NH2,HPLC purity>95%, mass spec-verified) were from GL Biochem (Shanghai, China). Mouse PAR-2 agonist peptide (mPAR-2-AP; NH2–Ser–Leu–Ile–Gly–Arg–Leu–NH2, HPLC purity>95%, mass spec-verified) was from NeoBioLab (MA, USA). Antibodies against pERK and pSrc were from Cell Signaling (Boston, MA, USA) and the antibody against α-tubulin was from Santa Cruz Biotechnology. TGFβ inhibitors SB-431542 hydrate and LY-2157299 were
purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and Axon Medchem (Groningen, The Netherlands), respectively.

**Cell culture and cell lines**

Panc02 and BxPC-3 cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA), whereas Miapaca-2, Panc-1, HEK293 (stably transfected with empty vector, pcDNA3.1: HEK cell Ctrl; or human PAR2 YFP-tag: hiPAR-2 HEK cells; a kind gift of Dr van’t Veer, Academic Medical Centre Amsterdam; manuscript in preparation) and Swiss3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen). All media were supplemented with 10% fetal bovine serum (FBS; Lonza, Switzerland), 1% penicillin–streptomycin and 1% L-glutamine (Lonza). Human lymphatic endothelial cells (HULECs; obtained from from Dr Pepper, University of Geneva) [23] and human umbilical vein endothelial cells (HUVECs) were cultured in flasks with 1.5% gelatin coating and maintained in ECM-2 medium containing various growth factors, according to the standard procedure suggested by the manufacturer (Lonza, Switzerland). Mouse lymphatic endothelial cells (MLECs; CellBiologics, C57-6092, Chicago, IL, USA) were cultured in gelatin-coated flasks (supplied by CellBiologics) and maintained in Complete Mouse Endothelial Cell Medium (M1168, CellBiologics). All cells were cultured in a humidified incubator at 5% CO2 and 37 °C.

**Collection of conditioned media**

BxPC-3, Miapaca-2, Panc-1 or Panc02 cells were serum-starved for 24 h, washed with PBS and subsequently incubated with DMEM or RPMI supplied with 10% FBS. HULECs or MLECs were cultured with or without 100 μM PAR-2-AP in refreshed complete EGM-2 medium or complete mouse endothelial cell medium. After 24 h, all media were collected,
centrifuged at 4000 rpm for 10 min, put through a 0.2 μm filter and stored at –20 °C.

**Western blot assay**

Cells were seeded at $1 \times 10^5$ cells/well onto six-well plates and grown to 70% confluence. After overnight incubation in serum-free medium, the cells were incubated with the indicated compounds for the indicated times, after which they were lysed in Laemmli lysis buffer and the lysates were incubated for 5 min at 95 °C. Cell extracts were resolved by SDS–PAGE (10%) and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% fat-free dried milk in TBS with 0.05% Tween (TBS-T) and incubated overnight at 4 °C with primary antibodies at 1:1000 dilutions in 1% BSA/TBS-T. After washing in TBS-T, the membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated) at 1:1000 dilutions in TBS-T for 1 h. Detection was performed by enhanced chemiluminescence on an LAS4000 imager.

**Tube formation assays**

Twenty-four-well plates were coated with 100 to 150 μl/well Matrigel (Geltrex™ Reduced Growth Factor Basement Membrane Matrix, Invitrogen) and incubated at 37 °C for at least 30 min, according to the manufacturer’s instructions. Next, HULECs, HUVECs, Murine Endothelial Cell Line (2H11s) or MLECs were seeded at a density of 35 000 cells/well and the plates were incubated at 37 °C. Tube formation was monitored every 2 h by bright-field microscopy up to 10 h. For quantitative assessment, cord-like structures were counted in five randomly selected fields/well at × 50 magnification, as described previously [24].
Statistical analysis

Differences between multiple groups were analyzed by two-way ANOVA and/or Tukey t-tests, using GraphPad Prism; p<0.05 was considered significant.

Results

PAR-2 is expressed in the stromal compartment of primary pancreatic tumours

To assess the potential importance of PAR-2 in the microenvironment of pancreatic cancer, we determined PAR-2 expression in a set of 13 human pancreatic ductal adenocarcinomas (PDACs; three sections/patient). In all tumours, PAR-2 was intensively expressed in tumour epithelial cells, whereas it was also expressed in the stromal compartment of the majority of the tumours (i.e. fibroblasts, endothelial cells and inflammatory cells). Figure 1A shows representative images of tumours in which PAR-2 is expressed in the stromal compartment at different intensities. As shown in Figure 1B, PAR-2 is also expressed in normal pancreatic tissue sections, although expression levels are low compared to tumour PAR-2 expression.

Genetic ablation of PAR-2 from the stromal compartment limits primary tumour growth

Due to the expression of PAR-2 in the stromal compartment of human pancreatic tumours, we addressed the possibility that PAR-2 in the stromal compartment may contribute to tumour development. To this end, we used a murine orthotopic pancreatic tumour model in which PAR-2-expressing pancreatic tumour cells are injected directly into the pancreas of wild-type or PAR-2-deficient mice (Figure 2A). At the moment of sacrifice (5 weeks after tumour cell injection), wild-type mice had large primary tumours with an average weight of 1.82±0.46 g and a volume of 2.75±0.67 cm³ (Figure 2B–D). Interestingly, primary tumours in PAR-2-deficient mice were
significantly smaller (1.10±0.42 cm³) and lighter (0.91±0.44 g) than in wild-type animals (Figure 2B–D). Histological examination of H&E-stained pancreatic cancer sections showed no gross difference between primary tumours from wild-type or PAR-2-deficient mice (Figure 2E). Tumour cells showed variable PAR-2 expression in both wild-type and PAR-2-deficient mice (Figure 2F), whereas endothelial cells and/or ductal cells expressed PAR-2 in wild-type mice but not in PAR-2-deficient mice (Figure 2F).

Figure 1. PAR-2 expression in tumour epithelial cells and the stromal compartment of primary pancreatic tumours (A). Paraffin sections obtained from patients with resectable pancreatic cancer were stained for PAR-2 (brown). Shown are representative pictures showing PAR-2
expression by tumour epithelia cells (yellow arrows) and stromal cells (blue arrows). The lower three panels show representative pictures of negative isotype controls. Pictures were taken at magnification 200x. (B) Representative pictures showing PAR-2 expression in normal (non-tumor containing) human pancreas ducts. Pictures were taken at magnification 400x.

**Figure 2.** PAR-2 in the stromal compartment potentiates pancreatic tumour growth. (A) Schematic representation of the experimental set-up. (B) Pancreatic tumours derived from wild type mice (top) or mice that lack PAR-2 in the host microenvironment (bottom). (C) Weight and (D) size of the tumours depicted in panel B. Indicated is the mean +/- SEM (n=7-8 per group) **:p<0.01. (E) Paraffin sections obtained from tumour bearing wild type (top) or PAR-2 deficient mice (bottom) stained with hematoxylin and eosin. Representative pictures were taken at magnification 100x or 200x, as indicated in the figure. (F) Representative pictures of PAR-2 expression in tumours derived from wild type and PAR-2 deficient mice. Endothelial cells and/or ducts are indicated with black arrows, whereas highly positive stained tumour cells are indicated with **. Pictures were taken at magnification 400x.
**Genetic ablation of PAR-2 from the stromal compartment limits angiogenesis**

The reduced primary pancreatic tumour growth in PAR-2-deficient mice may depend on diminished proliferation of tumour cells in a PAR-2-deficient environment. Immunohistological examination of tumour cell proliferation by Ki67 stainings indeed showed significant differences between tumours derived from wild-type and PAR-2-deficient mice (Figure 3A). The number of Ki67-positive cells was two-fold higher in wild-type animals than in PAR-2-deficient animals (Figure 3A, right).

PAR-2 plays a critical role in stimulating blood vessel formation during tumour angiogenesis [25] and angiogenesis is a hallmark of cancer progression and a prerequisite for tumour growth [26]. Consequently, we next assessed whether the reduced tumour growth and proliferation in PAR-2-deficient mice correlated with angiogenic changes. To this end, CD31 expression in pancreatic tumour sections of wild-type and PAR-2-deficient mice was compared. As shown in Figure 3B (right panels), the number of CD31-positive vessels in tumours from wild-type animals (19.14±3.87) was almost two-fold higher than the number of positive vessels in tumours from PAR-2-deficient animals (11.67±1.67).

To assess the direct effect of PAR-2 stimulation on tube formation, we next stimulated 2H11 or HUVEC endothelial cells with PAR-2-agonist peptides. Both hPAR-2-AP and mPAR-2-AP were validated by the level of induced Erk1/2 phosphorylation (a widely used surrogate marker for PAR-2 activation [27]) on PAR-2-expressing HEK cells and Swiss3T3 cells, respectively (see supplementary material, Figure S1). Interestingly, however, PAR-2 stimulation of both endothelial cell types using the agonist peptides did not induce tube formation (Figure 3C, D), suggesting a paracrine mode of action. Overall, these data suggest that PAR-2-driven angiogenesis explains, at least in part, the reduced tumour growth in PAR-2-deficient mice.
Figure 3. PAR-2 in the stromal compartment enhances tumour cell proliferation and induces angiogenesis. (A-B) Paraffin sections obtained from tumour bearing wild type or PAR-2 deficient mice stained for (A) Ki-67 (proliferation) and (B) CD-31 (blood vessel formation). Right panels show quantifications of the sections depicted in panels (A) and (B). Indicated is the mean+/−SEM (n=7-8 per group). **:p<0.01; ***:p<0.001. (C) Representative pictures of 2H11 cells cultured with or without mPAR-2 agonist peptide (100 µM). Right panel shows the quantification of the numbers of tubes (experiment performed three times in triplo). (D) Representative pictures of
HUVEC cells cultured with or without hPAR-2 agonist peptide (100 µM). Right panel shows the quantification of the numbers of tubes (experiment performed two times in triplo).

**PAR-2 in the stromal compartment limits lymph node metastasis**

The poor prognosis of pancreatic cancer patients is largely due to metastatic disease. Therefore, we next assessed the role of PAR-2 in the stromal compartment during metastasis. Interestingly, all PAR-2-deficient animals showed lymph node metastasis (lymph nodes were collected from mesentery sites), whereas only three of eight animals in the wild-type group showed metastasis to the lymph nodes. As shown in Figure 4A, B, the sizes of the tumours in the lymph nodes were significantly increased in PAR-2-deficient as compared to wild-type animals. In line with increased lymph node metastasis, metastasis to secondary organs, such as liver, colon, intestine and spleen, was also observed more frequently in the PAR-2-deficient animals (Table 1), whereas tumour cells seemed to invade secondary organs more aggressively in PAR-2-deficient than in wild-type animals.

**Table 1.** Percentage of wild type and PAR-2 deficient mice with metastasis in the different organs at the time of sacrifice. (7 to 8 mice per group.)

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<td>PAR-2 -/-</td>
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**Enhanced lymph node metastasis in PAR-2-deficient mice correlates with lymphangiogenesis**

Lymphangiogenesis is a prerequisite for lymph node metastasis and the density of lymph vessels correlates with lymph node metastasis in several cancer models [28–30]. Consequently, we next assessed the number of
LYVE-1-positive endothelial vessels in tumours derived from wild-type or PAR-2-deficient mice. As shown in Figure 4C, D, the number of intratumoural lymphatic vessels was increased in PAR-2-deficient mice as compared to wild-type mice. Importantly, PAR-2-deficient mice do not generally feature reduced lymphangiogenesis, as evident from similar levels of LYVE-1-positive vessels in the intestine and (non-tumourigenic) pancreas of wild-type and PAR-2-deficient mice (Figure 4E).

To elucidate the underlying mechanism by which PAR-2 limits lymphangiogenesis, we next performed tube-formation assays with MLECs and HULECs. Direct activation of PAR-2 in either MLECs (Figure 5A) or HULECs (Figure 5B) did not modify tube formation. Interestingly, however, conditioned medium from pancreatic cancer cells induced tube formation (Figure 5C, Panc02-induced tube formation of mLECs; Figure 5D, BxPC-3-induced tube formation of HULECs) and the number of tubes was even further increased when the lymphatic endothelial cells were pre-incubated with the PAR-2 antagonist P2pal-18S (Figure 5C, D). Finally, we showed that medium obtained from PAR-2-stimulated mLECs or HULECs (CM-MLECs or CM-HLEC, respectively) significantly reduced cancer cell-induced tube formation (Figure 5C, D).

To validate our findings, we next incubated HULECs with conditioned media from alternative pancreatic cancer cell lines (ieMiapaca-2 or Panc-1 cells), again showing that pancreatic cancer cells induce tube formation of HULECs, that PAR-2 inhibition further increases tube formation, and that medium from PAR-2-stimulated HULECs reduces pancreatic cancer cell-induced tube formation (Figure 5E, F). Overall, these data suggest that PAR-2 expression in lymphatic endothelial cells limits cancer cell-induced lymphangiogenesis.
Figure 4. PAR-2 in the stromal compartment inhibits lymphangiogenesis. (A) Lymph node metastasis in wild type mice (top) or mice that lack PAR-2 in the tumour microenvironment (bottom). (B) Tumour volume comparison between wild type and PAR-2−/− mice. (C) Lymphatic vessel density comparison between wild type and PAR-2−/− mice. (D) Immunohistochemistry images of wild type (WT) and PAR-2−/− mice showing lymphatic vessels. (E) Immunohistochemistry images of pancreas and intestine from wild type (WT) and PAR-2−/− mice.
(bottom). (B) Size of the metastases depicted in panel A. (C) Quantification of the number of LYVE-1 positive vessels in primary tumours obtained from wild type and PAR-2 deficient mice. Indicated is the mean+/−SEM (n=7-8 per group). **: p<0.01; ***: p<0.001. (D) Representative pictures for panel (C). (E) Representative pictures of LYVE-1 positive vessels in the pancreas and intestine of wild type and PAR-2 deficient control (non-tumour bearing) animals.

Figure 5. Inhibition of PAR-2 enhances lymphangiogenesis in in vitro tube formation assays. (A) Tube formation of MLECs with or without direct PAR-2 activation with 100 μM mPAR-2-AP. (B) Tube formation of HULECs with or without direct PAR-2 activation with 100 μM hPAR-2-AP. (C) Top panel: Incubation of MLECs with medium collected from PANC02 cells (CM) increases tube numbers as compared to incubation in control medium (Neg Ctrl). Middle panel: Tube formation
Discussion

Our main finding was that PAR-2 expression in the stromal compartment plays a dual role in pancreatic cancer development. We showed that PAR-2 in the stromal compartment potentiates primary tumour growth, whereas it limits lymph node metastasis and subsequent dissemination.

The potential importance of PAR-2 in the setting of pancreatic cancer has mainly focused on PAR-2 expression by cancer cells. Several studies have shown that pancreatic cancer cells express high levels of PAR-2 [19] and that activation of PAR-2 induces the proliferation [14, 31] and migration [16] of pancreatic cancer cells. Interestingly, however, the stromal compartment also plays a key role in pancreatic cancer development [32] and PAR-2 expression is significantly increased in stroma-rich tumour regions [19], suggesting a potential role of PAR-2 in non-tumour cells. Here, we showed that PAR-2 is abundantly expressed in the stromal compartment of human
pancreatic tumours (Figure 1). Importantly, we next showed that PAR-2 expression by cells composing the microenvironment is not an epiphenomenon of tumour growth, but actually contributes to tumour progression (Figure 2). Consistently, both the size and weight of tumours in mice that lacked PAR-2 in the stromal compartment were reduced by around 35% as compared to wild-type mice.

In line with the literature showing that PAR-2 drives angiogenesis during tumour development [33], primary tumours in wild-type mice are better vascularized than tumours in PAR-2-deficient animals (Figure 3). Interestingly, direct activation of PAR-2 on 2H11s or HUVECs did not induce tube formation, suggesting that PAR-2-dependent angiogenesis is not regulated by PAR-2 in endothelial cells. Actually, pancreatic cancer cells secrete VEGF after PAR-2 stimulation, thereby inducing tube formation of HUVECs in vitro [34] and, potentially, tumour angiogenesis in vivo. Considering the critical role of vascularization for tumour cell proliferation, our data suggest that (stromal) PAR-2-dependent angiogenesis drives primary pancreatic tumour growth. However, despite the fact that drug responses are related to reduced angiogenesis in pancreatic cancer [35], the importance of angiogenesis for pancreatic cancer is under debate [36], largely because of the fact that anti-angiogenic treatment with Sunitinib did not reduce tumour burden in pancreatic ductal adenocarcinoma [37].

Pancreatic cancer is a devastating disease with the worst survival outcome of all human cancers. Importantly, metastasis is a major cause of morbidity and mortality in pancreatic cancer patients, and we showed that PAR-2 may play a key role in pancreatic cancer metastasis. Certainly, enlarged lymph nodes loaded with tumour cells were observed in PAR-2-deficient animals as opposed to wild-type animals (Figure 4). Moreover, metastasis to distant organs (such as spleen and liver) was increased in PAR-2-deficient mice. Interestingly, previous experiments did not show a rate-limiting role of PAR-2 in metastasis. Experimental metastasis of B16F10 melanoma cells in PAR-2-deficient mice is indistinguishable from metastasis in wild-type
animals [38]. Moreover, PAR-2-deficient mice showed decreased tumour growth and metastasis in a spontaneous PyMT breast cancer model, although the difference in metastasis lost significance when subgroups of wild-type and PAR-2-deficient mice with similar tumour burdens were compared [39].

The increase in lymph node metastasis was accompanied by a significant increase in the number of lymph vessels in tumours of PAR-2-deficient mice (Figure 4). Subsequent in vitro experiments showed that PAR-2 expression in lymphatic endothelial cells truly limits lymphangiogenesis (Figure 5). Interestingly, direct activation of PAR-2 in lymphatic endothelial cells did not affect tube formation, but the inhibition of PAR-2 expression in lymphatic endothelial cells did potentiate cancer cell medium-induced tube formation (Figure 5). Based on these data, we propose a model in which cancer cells directly target lymphatic endothelial cells to induce angiogenesis (probably via secretion of VEGF-C or VEGF-D [40–43], but at the same time cancer cells secrete a PAR-2 agonist that acts upon lymphatic endothelial cells to secrete an inhibitor of cancer cell-induced lymphangiogenesis (Figure 6). In cases where lymphatic endothelial cells do not express PAR-2 (i.e. PAR-2-deficient mice), the inhibitor is not secreted, leading to tumour cell-induced lymphangiogenesis and subsequent lymph node metastasis.

The actual PAR-2 agonist secreted by the pancreatic cancer cells that induces inhibitor expression remains elusive at the moment, and is the subject of ongoing experiments. Importantly, trypsins, coagulation factors, tissue kallikreins and transmembrane proteases, such as matriptase, may all mediate PAR-2 cleavage [44] and any of these proteases may thus be the endogenous PAR-2 agonist. Particularly interestingly, KLK6 shows strong expression in pancreatic cancer and KLK6/KLK10 co-expression correlates with poor survival [45]. Moreover, PRSS3, a member of the trypsin family of serine proteases, is over-expressed in metastatic pancreatic cancer cells and PRSS3 expression levels correlated with metastasis of human pancreatic
cancers [46]. Finally, matriptase is significantly up-regulated in pancreatic ductal adenocarcinoma compared to normal pancreatic ducts.

Figure 6. Proposed model of PAR-2-dependent inhibition of tumour-induced lymphangiogenesis. Tumour cells secrete angiogenic factors like VEGF-C and –D inducing tube formation of lymphatic endothelial cells. In addition, pancreatic cancer cells secrete PAR-2 agonists (for instance, trypsine or matriptase) that act upon PAR-2 expressed on lymphatic endothelial cells leading to the secretion of an inhibitor of tumour cell-induced lymphangiogenesis.

Negative regulators of lymphangiogenesis are not particularly well described. Recently, however, it has been suggested that TGFβ may inhibit the generation of lymphatic vessels [47]. The addition of different TGFβ inhibitors (SB-431542 and LY-2157299) did not, however, increase tube numbers in cancer cell medium-stimulated HULECs (data not shown). The
nature of the PAR-2-induced inhibitor of lymphangiogenesis thus remains to be identified. Irrespective of the actual inhibitor, our data provide compelling evidence that PAR-2 plays a novel role in lymphangiogenesis and metastasis, which may have important implications; for instance, it is tempting to speculate that the dual role of PAR-2 in pancreatic cancer may (at least in part) explain the minimal effect of anticoagulant treatment on overall survival in pancreatic cancer patients [10]. Despite an overwhelming amount of preclinical data suggesting that blood coagulation may contribute to cancer progression [48–51], randomized clinical trials assessing the potential of anticoagulants in the treatment of cancer patients showed only small effects at best [52]. Obviously this is in part due to the type of patients (i.e. unselected patients with end-stage disease without any other treatment option), but our data suggest that the inhibition of TF/FVIIa- or FXa-induced PAR-2 signaling by anticoagulant treatment with subsequent increased lymph node metastasis may also contribute.

In the current study, we employed orthotopic pancreatic cancer cells in which murine PANC02 cells were grafted into the pancreas of immune competent wild-type or PAR-2-deficient mice. This orthotopic model, which is frequently used to study the interplay between tumour cells and their microenvironment [53–55], offers tissue-specific pathology and is generally deemed more clinically relevant than xenograft models [56,57]. Especially importantly, the number and distribution of blood and lymphatic vessels in the orthotopic PANC02 model are similar to those in patients (for representative images of human pancreatic cancer slides stained for CD31 and D2-40, see supplementary material, Figure S2). A limitation of the PANC02 cell line may be that it is not generally considered metastatic, as opposed to some of its derived cell lines [58]. This is particularly relevant in the setting of novel metastatic murine pancreatic cancer cell lines derived from genetically modified mouse models that develop pancreatic ductal adenocarcinoma similar to human disease (e.g. Rink-1 [59,60]). Overall, however, we feel that our orthotopic model is perfectly suited for studies
assessing (lymph) angiogenesis and subsequent metastasis, but future studies should obviously assess the general nature of our findings by analysing PAR-2-dependent lymph node metastasis in alternative tumour models.

We employed different human pancreatic cancer cell lines (i.e. BxPC-3, Panc-1 and MiaPaCa-2), which all accumulate different genetic alterations. The BxPC-3 cell line is wild-type for KRAS as opposed to Panc-1 and MiaPaCa-2 cells have mutant KRAS [61]. Moreover, MiaPaCa-2 and Panc-1 cells express low tissue-factor levels [62], whereas BxPC-3 cells express high tissue-factor levels [16]. Importantly, however, all three cell lines show similar effects in our in vitro experiments, thereby suggesting that the observed effects are of a more general nature and are not specific for certain tumour cells.

In conclusion, we show that PAR-2 in the stromal compartment promotes primary tumour growth but limits lymphangiogenesis and subsequent metastasis to the lymph nodes. These data point to a novel role of PAR-2 in cancer and strongly suggest that targeting PAR-2 (or its ligands) should be pursued with care.

**Acknowledgements**

We would like to thank Joost Daalhuisen and Marieke ten Brink for expert technical assistance. This study was supported by the Dutch Cancer Society (KWF; Grant No. AMC 2009-4324).
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Supplementary Figures

Figure S1. Validation of PAR-2 agonist peptides. Control transfected HEK cells (A) or PAR-2 transfected HEK cells (B) were stimulated with hPAR-2-AP (100 µM) after which Erk1/2 phosphorylation was assessed. (C-D) Erk1/2 phosphorylation of murine Swiss3T3 cells stimulated with hPAR-AP (C) or mPAR-2-AP. Alpha-tubulin served as loading control.

Figure S2. Representative pictures of human pancreatic cancer slides stained for CD31(A, Brown) and D2-40 (B, brown). Paraffin sections obtained from patients with resectable pancreatic cancer were stained for CD31 (A, Brown) and D2-40 (B, Brown). Three different biopsies from three different patients were randomly selected from the tissue array.