Nicotinamide phosphoribosyltransferase and prostaglandin H_2 synthase 2 are up-regulated in human pancreatic adenocarcinoma cells after stimulation with interleukin-1

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Abstract. Human pancreatic cancer is today an almost incurable disease with a 5-year survival rate of <5%. Chronic inflammation in the tumor region is often associated with cancer progression. In pancreatic tumors, the pro-inflammatory cytokine IL-1 has been found to affect the development of chemoresistance in this cancer type. In a search for new therapeutic targets we investigated the effect of this pro-inflammatory mediator on pancreatic cancer protein expression. Therefore, the human pancreatic adenocarcinoma cell line Colo357 was subjected to proteomic analysis after stimulation with IL-1 using 2D gel electrophoresis and mass spectrometry. We detected 11 spots as being differentially expressed after stimulation with IL-1 representing 11 different proteins. Among them, nicotinamide phosphoribosyltransferase (NAMPT) and prostaglandin H_2 synthase 2 (PGHS-2) are crucial proteins whose expression in Colo357 is increased by IL-1. This study is the first one demonstrating an up-regulation of NAMPT in a tumor model for human pancreatic cancer. Several clinical trials using selective PGHS-2 or NAMPT inhibitors alone did not lead to an improvement in clinical outcome. Against the background of a high cardiovascular risk associated with PGHS-2-specific pharmacological inhibitors, we recommend a combinatory treatment with selective NAMPT and PGHS-2 inhibitors. This might overcome the limitations associated with PGHS-2 inhibitors since agents at low doses and with complementary mechanisms will be used. Such combined administration should positively affect the balance between risk and benefit in fighting the interplay of tumor-associated pancreatic inflammation and carcinogenesis in high-risk patients with pancreatic neoplasia.

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Introduction

Pancreatic cancer is the fourth cause of cancer-related mortality in the USA and the sixth one in Europe with more than 250,000 patients estimated to die of the disease worldwide (1). This cancer entity is one of the most deadly of all malignancies with a death to incidence ratio approaching one making mortality rate a robust approximation of incidence for pancreatic cancer (2). The incidence of pancreatic cancer correlates with increasing age reaching a peak in the 65-75-years old age group (3). Approximately three-fourths of patients will die within one year of diagnosis and <5% survive up to 5 years after diagnosis (4). The poor outcome of pancreatic cancer is reflected by non-resectable primary lesions, locally advanced tumors, and a high metastatic potential (5). Since only a few patients benefit from classical adjuvant chemo- and radiotherapy, complete surgical resection is the superior treatment modality for patients with resectable disease. Unfortunately, the majority of pancreatic cancer patients present with locally advanced or metastatic tumors are not amenable for curative surgery. Only a disappointing 15% of patients at the time of diagnosis are eligible to undergo surgical excision, and even patients who have undergone such curative resection often die of recurrent carcinoma (6).

In recent years, the association of chronic inflammation with the development of cancer has been rediscovered (reviewed in ref. 7). Pancreatic inflammation has been shown to play a key role in the development of pancreatic malignancy obviously mediated by the release of reactive oxygen intermediates, cytokines and the induction of pro-inflammatory signaling cascades (8). Patients suffering from hereditary pancreatitis have a 50 times higher risk of developing pancreatic carcinoma compared to healthy individuals (9). Convincingly, 40% of patients with hereditary pancreatitis are diagnosed with pancreatic cancer within the seventh life decade (10). The persistent expression of pro-inflammatory cytokines at tumor sites exert pleiotropic effects on the malignant process. On the one hand, they affect carcinogenesis and malignant transformation, tumor growth, invasion and metastases, on the other hand they activate immune effector mechanisms limiting tumor growth. Of special relevance to the malignant process is IL-1

that has been found to affect development of chemoresistance in pancreatic tumors (11). Moreover, IL-1 induces the expression of several pro-inflammatory genes in diverse inflammatory and stromal cells promoting tumor growth and invasiveness. In stromal and malignant cells, IL-1 induces secretion of angiogenic as well as growth- and invasiveness-promoting factors contributing to tumor progression (12). In contrast, anti-tumor immunity, reduced metastasis and increased survival rates can also be observed (13,14). Less is known about the effects of IL-1 on the process of pancreatic carcinogenesis. Recent data indicate a promoting activity on angiogenesis during tumor progression (15). IL-1 signal transduction is initiated by binding of either form of IL-1 to IL-1 receptor type I (IL-1RI), which undergoes a conformational change allowing the IL-1 receptor accessory protein (IL-1RAcP) to recognize the ligated IL-1RI. IL-1RAcP does not recognize IL-1 but represents an essential component in the IL-1 signaling pathway (16,17). Ligand-mediated heterodimerization of the receptor complex leads to the induction of several intracellular signaling pathways culminating in the activation of a great variety of transcription factors including NF-κB (18). More recently, it has become clear that NF-κB signaling plays a critical role in cancer development and progression (19).

Since pancreatic carcinoma is largely refractory to conventional therapies, there is a strong medical need for the development of novel therapeutic strategies. In a search for putative therapeutic targets we investigated the effect of pro-inflammatory IL-1 on pancreatic cancer protein expression. For this purpose, the human pancreatic adenocarcinoma cell line Colo357 was subjected to proteomic analysis after stimulation with IL-1 using 2D gel electrophoresis and mass spectrometry in order to determine alternately expressed proteins. Mass spectrometric analysis of tryptic peptides identified two proteins found in more abundance in these cells as nicotinamide phosporibosyltransferase and prostaglandin H_2 synthase 2 whose expression is up-regulated under tumor-associated pancreatic inflammatory conditions. Thus, our data might have future clinical implications with respect to the development of novel approaches as an adjuvant therapy in high-risk patients with human pancreatic carcinoma.

Materials and methods

Cell culture and stimulation. The human pancreatic adenocarcinoma cell line Colo357, kindly provided by Dr G. Multhoff (Klinikum rechts der Isar, Abteilung für Radiotherapie und Radioonkologie, Technische Universität München, Germany) was frozen in aliquots of $2.5x10^6$ cells/ml in liquid nitrogen. After thawing, Colo357 cells were seeded at a density of $2.5x10^4$ cells/cm^2 and maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate in the presence of 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) at 37˚C in 5% CO_2. Single-cell suspensions were derived by short-term (<2 min) trypsin-EDTA treatment (all cell culture reagents were purchased from PAA Laboratories, Cölbe, Germany, unless otherwise stated). Before stimulation viability of Colo357 cells, as assessed by the trypan-blue exclusion assay, was always >95%. For stimulation experiments $7x10^5$ Colo357 cells were seeded in 6-cm culture plates and, after a recovery phase of at least 16 h, stimulated with 1 ng/ml of recombinant human IL-1ß (Pan Biotech, Aidenbach, Germany) for the indicated time-points.

Protein extraction. For proteome analysis total protein was extracted from Colo357 cells stimulated with or without 1 ng/ml of IL-1, for 24 h by TriFast® Reagent (PEQLAB Biotechnology GmbH, Erlangen, Germany). After washing with 250 mM sucrose, 10 mM Tris-HCl (pH 7.0) the cells were lyzed directly in a culture dish by adding of TriFast reagent. Cells were scraped after 5 min of incubation and the cell lysate passed several times through a pipette. Chloroform was added to the homogenate which was mixed thoroughly for 15 sec and incubated for 3 min at room temperature (RT). The phases were separated by centrifugation at 12,000 x g for 15 min at 4˚C. RNA remaining in the aqueous phase was precipitated with isopropanol and used for mRNA analysis in quantitative real-time PCR. DNA remaining in the interphase and phenol phase was recovered by ethanol precipitation and centrifugation at 12,000 x g for 1 min at 4˚C. Proteins in the supernatant were then precipitated for 10 min by isopropanol followed by centrifugation at 12,000 x g for 5 min at 4˚C. After precipitation the protein pellet was washed, vacuum dried and resuspended directly in lysis buffer [8 M urea, 2 M thiourea, 4% (w/v) Chaps, 40 mM Tris-base, 10 mM DTT] for DeStreak® rehydration [GE Healthcare, Uppsala, Sweden] (20).

Two-dimensional gel electrophoresis (2-DE). Isoelectric focussing (IEF) was performed with 24-cm pH 3-10 NL IPG strips according to the manufacturer's instructions with minor modifications to improve resolution. Briefly, for preparative 2-DE 500 μg of protein lysate were made up to 450 μl with rehydration buffer [8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1.2% DeStreak with 0.5% (v/v) IPG buffer pH 3-10 NL; GE Healthcare] and used to passively rehydrate each IPG strip (24-cm Ready Strip pH 3-10 NL; GE Healthcare) overnight. Proteins were separated by the Protean® IEF Cell (Bio-Rad, Hercules, CA, USA) using a programmed voltage gradient at 20˚C and a maximum of 50 mA per strip. The focusing was started at 500 V for 30 min, increased to 1,000 V in 1 h with linear gradient, then increased to 10,000 V with a linear gradient and kept constant until a total run of 50 kVh. After IEF, the IPG strips were equilibrated for 15 min in equilibration buffer 1 [375 mM Tris-HCl, pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 130 mM DTT] and then transferred to equilibration buffer 2 containing 135 mM iodoacetamide instead of 130 mM DTT for 15 min with constant shaking. The equilibrated strip was applied onto the top of a 12.5% uniform SDS-PAGE gel and sealed with 1% (w/v) agarose prepared in SDS-Tris-glycine buffer with trace amounts of bromophenol blue as a tracking dye to monitor electrophoresis. Electrophoresis was performed using the Protean Plus Dodeca™ Cell System (Bio-Rad) with constant voltage of 80 V at 20˚C. Preparative gels were stained with Roti®-Blue (Carl Roth GmbH, Karlsruhe, Germany). Therefore, the gels were fixed after electrophoresis in 40% (v/v) ethanol/15% (v/v) acetic acid for 4 h with constant shaking and then soaked

with colloidal staining solution overnight and destained in 20% (v/v) methanol to remove background staining.

Image analysis. Stained gels were digitized with a scanner (Expression 1600 Pro, Epson), and gel images were analyzed using the Delta2D software package v3.6 final9 (Decodon, Greifswald, Germany). Spot detection, matching and quantification were performed with a total of 3 gel images per group and each of the three independent experiments. Running differences between the gels were compensated using the exact warping mode guided by individually set match vectors connecting corresponding spots in two gel images. Firstly, warping was performed within each treatment group, then the master gels of each treatment group were warped together. Performing image fusion, the 18 single gel images were combined to a fused image containing all spots present in any of the gels. This fused image was used for spot detection and editing. From the fused image the spot boundaries were transferred to each single gel image providing unique spot matching and quantification. Spots were selected for protein identification if their mean % volume in the gels of the sample group was significantly different ($p<0.05$) by a factor of >1.5 from their mean % volume in the gels of the control group.

Preparation of peptide mixtures for ESI-MS. Protein identification was performed as described recently (21). For identification by mass spectrometry, the protein spots were manually excised from colloidal Coomassie Brilliant Blue-stained 2-D gels. They were washed and dehydrated. The digest was allowed to proceed by adding 20 μl of trypsin solution (10 ng/μl trypsin in 20 mM ammonium bicarbonate) overnight at 37˚C. Two peptide extractions were done by adding 20 μl of 1% (v/v) acetic acid/50% ACN and subsequent incubation for 30 min. Both peptide-containing supernatants were combined, and the volume was reduced to 10 μl and transferred to microvials for mass spectrometric analysis. HPLC separation was performed on an UltiMate™ System (LC Packings, Amsterdam, The Netherlands) coupled via a nano-LC inlet (New Objective, Woburn, MA, USA) to QTOF mass spectrometer (Q-Star Pulsar i, Applied Biosystems, Foster City, CA, USA) equipped with a nano-electrospray source (Protana, Odense, Denmark). Peptide solutions were loaded and desalted on a reversed-phase precolumn and separated via a reversed-phase nano-column. The eluted peptides were analyzed by MS/MS. All peptides in a mass range from 302 to 1400 with an intensity of at least 5 counts were fragmented, and an MS/MS spectrum was recorded in positive detection mode. The resulting MS/MS data were analyzed with the Bioanalyst™ Software (Applied Biosystems) and an integrated Mascot script. For database searches the Mascot search engine (Matrix Science Ltd., London, UK) was used with a SwissProt rel.54.6 database. Proteins were regarded as identified when at least 2 peptides were sequenced with an individual ion score of >28 ($p<0.05$).

Preparation of peptide mixtures for MALDI-MS. Proteins were excised from Colloidal Coomassie Brilliant Blue-stained 2-DE gels using a spot cutter (Proteome Works™ , Bio-Rad). Digestion with trypsin and subsequent spotting of peptide solutions onto the MALDI-targets were performed automatically in the Ettan Spot Handling Workstation (GE Healthcare). The MALDI-TOF measurement of spotted peptide solutions was carried out on a 4800 MALDI-TOF/TOFTM Analyzer (Applied Biosystems). The spectra were recorded in reflector mode in a mass range from 800 to 3,700 Da with an internal one-point-calibration on the autolytic fragment of trypsin. Additionally MALDI-TOF/TOF analysis was performed for the 5 strongest peaks of the TOF-spectrum after subtraction of peaks corresponding to background or trypsin fragments. After calibration a combined database search of MS and MS/MS measurements was performed using the GPS Explorer software v3.5 (Applied Biosystems). Peak lists were compared with the SwissProt rel.54.6 restricted to human taxonomy using the Mascot search engine 2.1 (Matrix Science Ltd.). Peptide mixtures that yielded at least twice a mowse score of at least 55 for SwissProt results were regarded as positive identifications (20).

Analysis of mRNA expression by quantitative RT-PCR. Total RNA was extracted from cultured Colo357 cells using TriFast Reagent (PEQLAB Biotechnologie GmbH) according to the manufacturer's instructions. RNA quantity was assessed by UV spectrophotometry. Reverse transcription was performed with 1 μg of RNA and oligo(dT) primer using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. PCR was carried out as a TaqMan method using the Bio-Rad CFX96 cycler and white 96-well reaction plates (Biozym, Hessisch Oldendorf, Germany). All real-time PCR reactions were run in a reaction volume of 10 μl, including 5 μl of 2x SensiMix dT (Quantace, London, UK), 0.8 μl cDNA, 500 nM of each primer and 200 nM probe using the following cycling conditions: 95˚C for 10 min followed by 44 cycles at 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 1 sec. The fluorescence signal was monitored during the 72˚C step. Each sample including blank controls were run in triplicate in three different PCR assays. PCR primers and 5'-FAM-3'-TAMRA-labelled probes were designed and synthesized by Microsynth (Balgach, Switzerland). For detailed sequence information see Table I. The amplicon length ranged between 95 and 136 bp. All primer pairs used in this study are located on different exons. For relative quantification, cDNA dilution series were run to set up a standard curve. Data analysis was performed with the CFX Manager™ software which employs a $\Delta\Delta C(t)$ relative quantification algorithm with PCR efficiency correction and single reference gene normalization.

Western blot analysis. Protein samples of 15 μg were separated by 12.5% SDS-PAGE. Fractionated proteins were transferred electrophoretically onto nitrocellulose membranes and blocked with 10% (v/v) Roti-Block (Carl Roth GmbH). Primary antibodies used were rabbit anti-human PGH synthase 2 (1:2,000; Acris Antibodies, Herford, Germany), mouse anti-human NAMPT (1:2,500; Alexis Biochemicals, San Diego, CA, USA) and rabbit anti-human GAPDH (1:5,000) from LabFrontiers Co. Ltd. (Seoul, Korea). Membranes were incubated with the primary antibodies for 1 h at RT or overnight at 4˚C in TBST consisting of TBS/0.1% (v/v) Tween-20/5% (w/v) BSA, washed 3x10 min with TBST and subsequently incubated for 1 h at RT with HRP-conjugated rabbit-IgG (1:10,000)

Table I. Sequences for primer and probes used in real-time PCR assays.

Gene/NIH accession no.	Primer/probe	Amplicon (bp)
PGHS-2/NM_000963	For: 5'-TGG AAC ATG GAA TTA CCC AGT TTG-3' Rev: 5'-TTT CTG TAC TGC GGG TGG AAC-3' Probe: 5'-CCT ACC ACC AGC AAC CCT GCC AGC-3'	95
NAMPT/NM_005746	For: 5'-GAG TTA TTC AAG GGG ATG GAG TAG-3' Rev: 5'-AGA TCT CTT GTC AAC TTC TGT AGC-3' Probe: 5'-AAC CTC CAC CAG AAC CGA AGG CAA-3'	136
RPLP0/NM_001002	For: 5'-GGG AAT GTG GGC TTT GTG TTC-3' Rev: 5'-TGG CAC AGT GAC TTC ACA TGG-3' Probe: 5'-CAA TGG CAC CAG CAC GGG CAG CA-3'	123

For, forward; Rev, reverse; RPLP0, ribosomal phosphoprotein, large, P0.

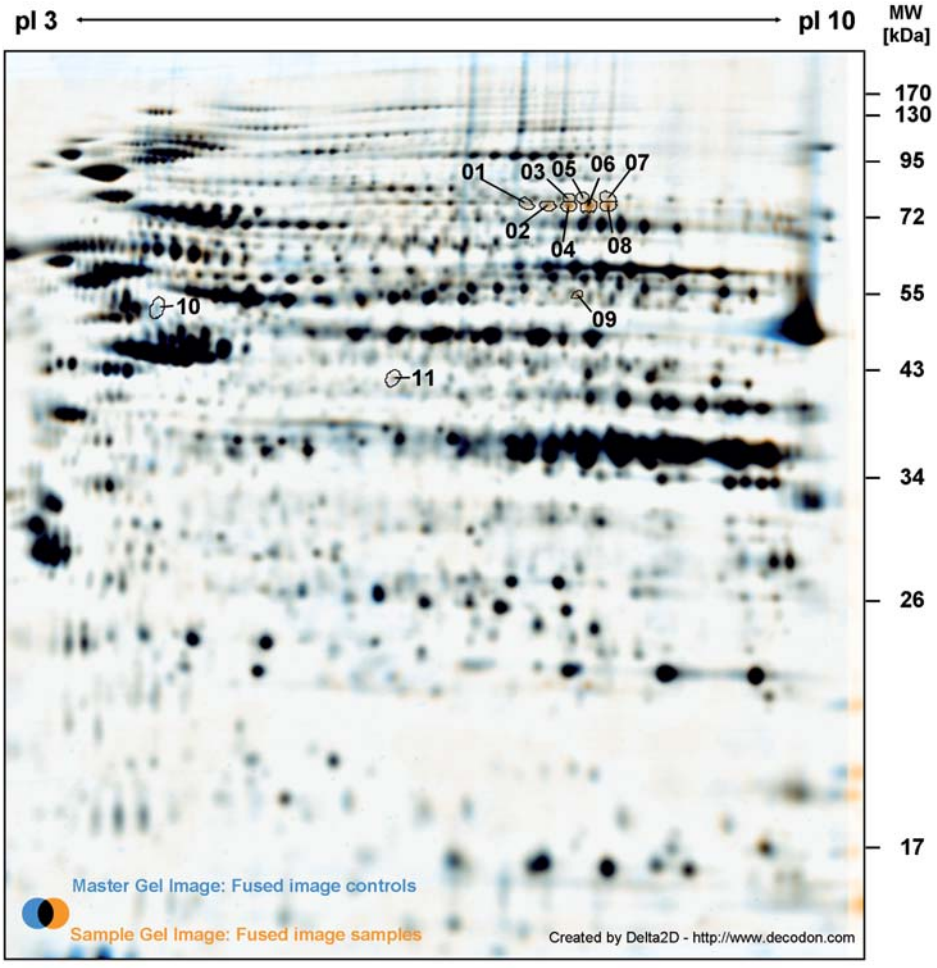

Figure 1. Dual channel image of fused sample gel images (orange) warped to fused control gel images (blue) representing protein patterns obtained by 2-DE derived from control and IL-1-treated Colo357 human pancreatic adenocarcinoma cells. Cells were incubated for 24 h in the presence or absence of 1 ng/ml of recombinant human IL-1ß. After preparation of cell lysates, proteins were resolved by IEF over the pI range 3-10, followed by 12.5% SDS-PAGE and visualized by Roti-Blue staining. Significantly differentially expressed proteins are labelled by numbers.

and mouse-IgG (1:5,000), respectively (both from Cell Signaling Technology, Boston, MA, USA). The presence of the proteins identified by MS/MS was revealed using the LumiGLO® chemiluminescent substrate (Cell Signaling Technology). For densitometric analysis films were scanned, and the intensity of the corresponding bands was quantified

Table II. Measurement of 11 protein spots by mass spectrometry.

Spot	ID[a]	Name	Peptides[b]	Score[c]	MW[d]	pI[e]	Ratio
1		Not identified	-	-	-	-	1.55
2	P02545	Lamin-A/C	19	92	74095	6.57	1.98
	P35354	Prostaglandin H_2 synthase 2	13	78	68952	7.02	
3		Not identified	-	-	-	-	1.9
4	P35354	Prostaglandin H_2 synthase 2	18	159	68952	7.02	3.17
5		Not identified	-	-	-	-	1.67
6	P35354	Prostaglandin H_2 synthase 2	21	210	68952	7.02	3.5
	P02545	Lamin-A/C	18	180	74095	6.57	
	P43304	Glycerol-3-phosphate dehydrogenase. Mitochondrial precursor	12	120	80834	7.23	
7		Not identified	-	-	-	-	2.17
8	P35354	Prostaglandin H_2 synthase 2	4	87	68952	7.02	3.4
9	P43490	Nicotinamide phosphoribosyltransferase	30	835	55487	6.69	1.66
	P00846	ATP synthase subunit alpha. Mitochondrial precursor	10	318	59714	10.09	
	P00390	Glutathione reductase. Mitochondrial precursor	9	202	56221	8.74	
	Q16851	UTP-glucose-1-phosphate uridylyltransferase 2	13	167	56905	7.69	
10		Not identified	-	-	-	-	-1.67
11	P30740	Leukocyte elastase inhibitor	19	1015	42829	5.9	1.76
	Q15019	Septin-2	13	698	41689	6.15	
	Q15366	Poly-(rC)-binding protein 2	6	360	38955	6.33	
	O43684	Mitotic checkpoint protein BUB3	2	141	37587	6.36	

Spots were cut from Coomassie gels and proteins submitted to in-gel digestion by trypsin. Fragment masses and partial sequences were obtained by MALDI-TOFT/TOF or ESI-MS/MS. Protein identification was performed by fragment-mass and sequence comparison with SwissProt database 54.6; cut-off score >55 for MALDI-TOF/TOF or cut-off score >28 for ESI-MS/MS with $p<0.05$. [a]SwissProt ID; [b]number of matched peptides; [c]identification score; [d]theoretical molecular weight; [e]theoretical isoelectric point.

using Kodak 1D Image Analysis Software and standardized to GAPDH.

Statistical analysis. Statistical analysis of Western blots and real-time PCR was performed using GraphPad Prism 3.0 software package (GraphPad Software, Inc., La Jolla, CA, USA). Standardized net intensities of sample bands were divided by control band intensities. Resulting relative expression values for PGHS-2 and NAMPT were analyzed in a two-tailed Mann-Whitney test against $\mu_0 = 1$ as the theoretical mean of controls and are expressed as means ± SEM. Differences were considered as significant for $p<0.05$.

Results

Detection of protein spots on 2-DE gels. Protein expression was compared between untreated and IL-1-stimulated Colo357 human pancreatic adenocarcinoma cells. At least 956 protein spots were matched on each 2-DE gel. The differential expression of paired control and IL-1-stimulated cells was detected by Delta2D software. Eleven spots were modulated in the presence of IL-1 >1.5-fold with a $p<0.05$. Among them, 10 spots showed up-regulation (spots 1-9, spot 11) whereas one spot (spot 10) showed down-regulation at the protein level. All 11 spots were chosen for cutting off and subsequent mass spectrometric analysis (Fig. 1).

Protein identification. As given in Table II, MALDI-TOF-MS/MS and ESI-MS/MS identified 6 spots out of 11 as protein-bearing spots. In four spots two or more proteins could be identified, whereas the remaining 2 spots harbored one protein only. Consequently, the same protein was sometimes found in multiple spots. A total of 11 different proteins was identified. Among them, nicotinamide phospho-

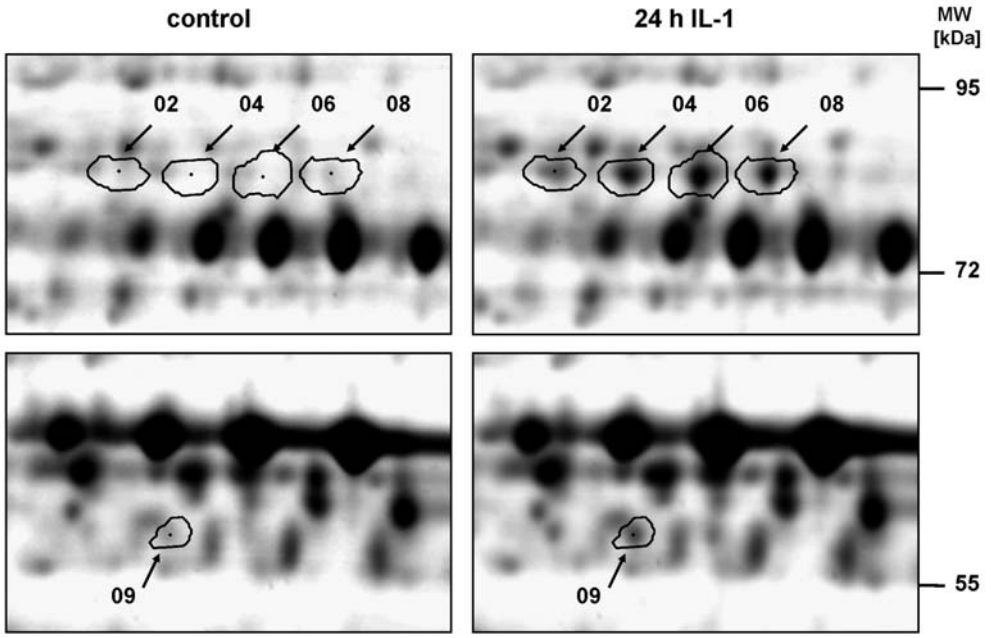

Figure 2. Enlarged regions of Roti-Blue-stained gel images indicating PGHS-2 (upper panel) and NAMPT (lower panel). Spot numbers correspond to those given in Fig. 1.

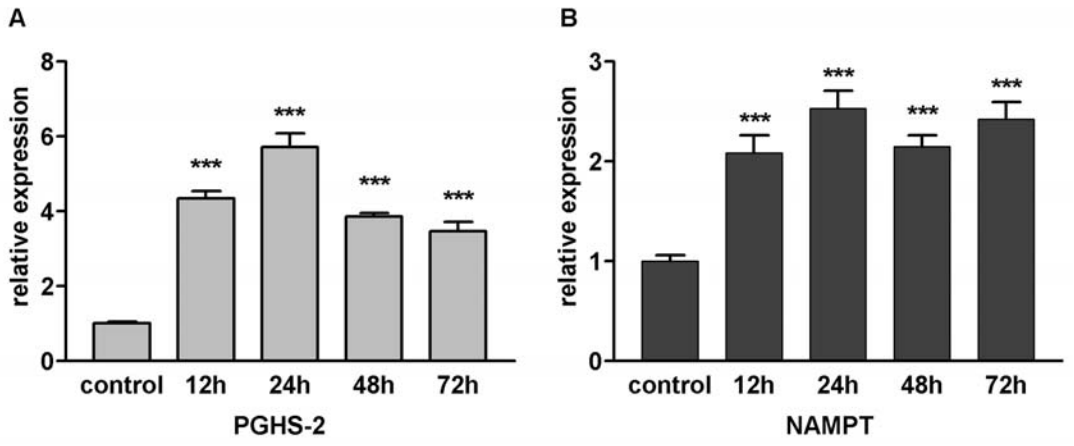

Figure 3. Effect of IL-1 on PGHS-2 and NAMPT expression in human pancreatic adenocarcinoma cells. Colo357 cells were treated with or without 1 ng/ml of recombinant human IL-1ß for the indicated time-points, and mRNA expression of PGHS-2 and NAMPT was analyzed by quantitative real-time PCR. mRNA expression of the untreated controls was set as one. Data represent means ± SEM from four separate experiments. $^{***}p<0.001$ compared to control.

ribosyltransferase (NAMPT) and prostaglandin H_2 synthase 2 (PGHS-2) were found in more abundance in IL-1-stimulated Colo357 cells compared to the untreated controls. NAMPT was identified in one spot (spot 09), whereas PGHS-2 was found in four different spots (spots 02,04,06,08) as demonstrated in Fig. 2. Other proteins, whose expression was up-regulated by IL-1, are as follows: leukocyte elastase inhibitor (LEI), septin-2 (SEPT2), poly-(rC)-binding protein 2 (PCBP2) and mitotic checkpoint protein BUB3 (BUB3) in spot 11; mitochondrial precursor of glycerol-3-phosphate dehydrogenase (GPDM) in spot 06 as well as mitochondrial precursor of ATP synthase subunit alpha (ATP6), mitochondrial precursor of glutathione reductase (GSHR) and UTP-glucose-1-phosphate uridylyltransferase 2 (UGPA) in spot 09. We also identified lamin-A and lamin-C in spots 02+06, respectively. These molecules represent isoforms of the same protein generated by alternative splicing from the corresponding mRNA. However, our proteomic approach did not allow to differentiate between these two isoforms.

Expression profiles of NAMPT and PGHS-2. Since NAMPT and PGHS-2 had been characterized previously as playing a crucial role in malignancy, they were selected for further analyses. For this purpose, we treated Colo357 cells with or without IL-1 and analyzed changes in the expression levels of NAMPT and PGHS-2 by quantitative RT-PCR. As demonstrated in Fig. 3, IL-1 induced a strong up-regulation of the NAMPT and PGHS-2 mRNA expression in a time-dependent manner. PGHS-2 expression reached a maximum after 24 h in the presence of IL-1 and declined thereafter. In

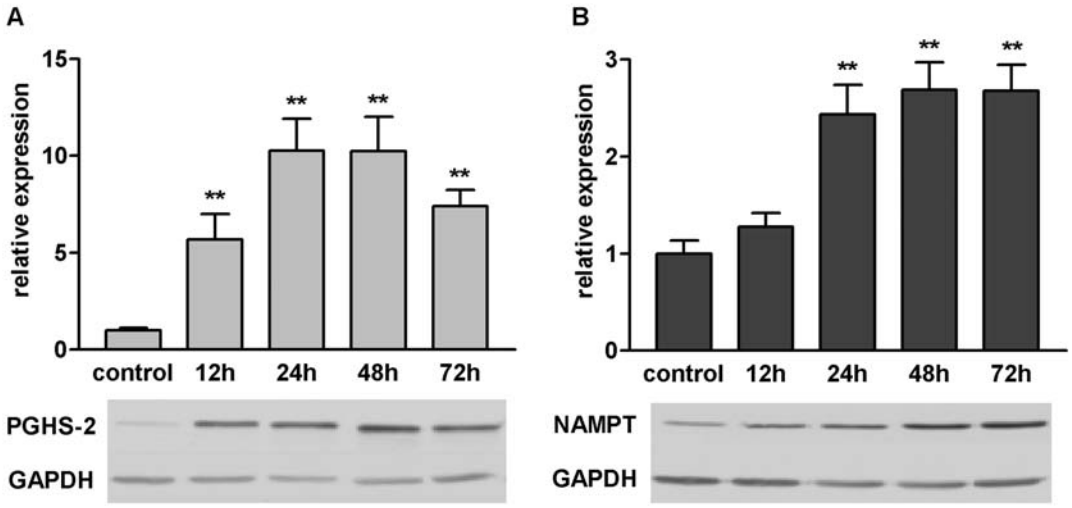

Figure 4. Time-dependent up-regulation of PGHS-2 and NAMPT in Colo357 cells after IL-1 stimulation. Whole-cell lysates of control and IL-1-treated Colo357 cells were separated by 12.5% SDS-PAGE and visualized by Western blotting. Data were quantified by densitometric analyses and standardized to the GAPDH response. They are expressed as means ± SEM and were derived from six separate experiments. $^{**}p<0.01$ compared to control.

contrast, NAMPT expression reached a plateau during this time and remained constantly for a further 48 h. In order to test whether this time course in IL-1-induced gene expression of NAMPT and PGHS-2 might affect protein expression profiles in a comparable way, we determined expression patterns of both molecules at the protein level in lysates of Colo357 after stimulation with IL-1. Consistent with the results obtained by quantitative real-time PCR, IL-1 was found to induce a massive up-regulation of the NAMPT and PGHS-2 protein expression in a time-dependent manner as determined by Western blot analyses. Whereas PGHS-2 protein expression peaked within 24 h of IL-1 stimulation followed by a rapid degradation, NAMPT protein expression reached a maximum after one day and remained unaffected during the following observation period (Fig. 4).

Discussion

Pancreatic cancer is one of the most fatal among all solid malignancies. Retrospective studies have revealed a global increase in the mortality rate of pancreatic carcinoma. This trend of an increasing incidence, together with the poor prognosis of the disease, emphasizes the need to develop novel therapeutic approaches. In the present study we therefore aimed to identify putative target molecules that could lead to an improved treatment and diagnosis, respectively. For these studies, the human pancreatic adenocarcinoma cell line Colo357 was chosen. Colo357 was originally derived from a lymph node metastasis of a human non-endocrine pancreatic cancer and characterized as being moderately differentiated (22). Colo357 harbors point mutations in the K-*ras* proto-oncogene (23), a common feature of pancreatic ductal neoplasms obviously contributing to the malignant phenotype, since activating point mutations in the K-*ras* proto-oncogene are encountered in >90% of pancreatic carcinomas (24).

In an unpublished observation by our group, Colo357 was found to dramatically up-regulate tumorigenic factors in the presence of the pro-inflammatory cytokine IL-1. In order to identify differentially expressed molecular targets under tumor-associated pancreatic inflammatory conditions, Colo357 was used in a quantitative proteomic approach. A systemic analysis of the aberrantly expressed proteome in this human pancreatic adenocarcinoma cell line identified 11 proteins whose expression is up-regulated under tumor-associated inflammatory conditions. Not surprisingly, some of them such as the mitotic checkpoint protein BUB3, lamin-A/C and septin-2 (SEPT2) are evolutionarily conserved proteins with essential functions in cytokinesis and more subtle roles throughout the cell cycle.

BUB3 belongs to the major spindle checkpoint proteins over-expressed in several breast and gastric cancer cells (25,26). The strong correlation of BUB3 expression and tumor cell proliferation in gastric cancer suggests that BUB3 over-expression is a proliferation-dependent phenomenon. Based on these findings one can assume that the IL-1-induced up-regulation of BUB3 in Colo357 might contribute to the high proliferative response of human pancreatic cancer cells supporting tumor growth and invasiveness.

SEPT2 is a member of the conserved family of septins. These GTP-binding proteins have several roles during cell division, cytoskeletal organization and membrane-remodelling events. The role of SEPT2 in tumorigenesis is controversely discussed in the literature. Whereas SEPT2 has been found as being down-regulated in human glioblastoma and breast cancer cells (27,28), an increased expression of SEPT2 in renal cell carcinoma was shown to be a common event (29). From these data we speculate that septins might belong to a class of cancer critical molecules where alterations in the expression profile may underline their role in neoplasia.

Lamin A/C comprises a group of structurally related proteins generated by alternative splicing. Lamins are members of a protein family that are the main structural elements of the nuclear envelope in eukaryotic cells. Unlike lamin C, A-type lamins are the main components of the nuclear lamina and represent type V intermediate filament proteins encoded by the gene *LMNA* (30). Mutations in *LMNA* give rise to diverse degenerative diseases related to premature ageing. Otherwise A-type lamins also affect the

activity of the retinoblastoma protein (pRb) and oncogenes such as ß-catenin. Consequently, it has been speculated that the expression of A-type lamins may also affect tumor progression. Willis *et al* observed that lamin-A is expressed in colonic stem cells and that patients with A-type lamin-expressing tumors have a significantly worse prognosis than patients with A-type lamin-negative tumors. From these observations we hypothesize that up-regulation of lamin A might also represent a putative marker for the poor prognosis in pancreatic carcinoma.

Poly-(rC)-binding protein 2 (PCBP2), one of the proteins over-expressed in Colo357 under the influence of IL-1, belongs to a group of heterogeneous nuclear ribonucleoproteins which bind RNA during transcription and take part in the process of splicing. These proteins indicate high synthesis rates of the expressing cells. The analysis of several gene expression profiling studies demonstrated a consistently elevated expression of the PCBP2-mRNA in metastatic prostate cancer (31). A meta-analysis of human microarray information with an algorithm discerning similarities in gene-regulatory profiles identified two molecules, PCBP2 and the stress kinase-interacting protein 1 (SIN1), as being generally co-regulated with large numbers of genes implicated in both, cell survival and cellular stress responses including RNA translation and processing (32). SIN1 is a scaffold protein that organizes anti-apoptotic stress responses, whereas PCBP2, its binding partner, provides for the selective expression of cell survival factors through posttranslational events.

Another protein up-regulated by IL-1 and co-chromatographing with PCBP2, BUB3, and SEPT2 in Colo357 was identified as leucocyte elastase inhibitor LEI. LEI belongs to the ovalbumin subgroup of serpins (serine protease inhibitors). Most of them inhibit target proteases and present diversified functions (33). The anti-protease activity of LEI is essential for its anti-apoptotic effect obviously mediated via cleavage of procaspase-8 (34). LEI over-expression slows down this cleavage thus protecting cells from apoptosis suggesting that high expression of LEI in pancreatic carcinoma cells might be jointly responsible for the chemoresistance of human pancreatic cancer. In contrast, studies using papilloma virus-transformed keratinocytes indicated a more likely down-regulation of LEI (35) obviously reflecting peculiarities of these tumor cells or influence of the microenvironment.

In the present study an IL-1-induced up-regulation of the mitochondrial precursor of glycerol-3-phosphate dehydrogenase (GPDM) could also be observed. The mature protein is the rate-limiting enzyme in the glycerol phosphate shuttle, which is thought to play an important role in cells requiring an active glycolytic pathway (36). Over-expression of enzymes involved in the glycolytic pathway is a common feature of cancerous tissues. Increased glycolysis in cancer cells has been regarded as the result of intratumoral hypoxia and is possibly associated with tumor invasion, metastasis or resistance to therapy (37). As reported previously, GPDM participates in the reoxidation of cytosolic NADH by delivering reducing equivalents from this molecule into the electron transport chain, thus sustaining glycolysis (38). It is well established that most malignant cells produce high levels of reactive oxygen species (ROS) compared to normal cells. In this context Chowdhury *et al* evaluated the glycerophosphate-dependent ROS production in prostate cancer cells. The author analyses revealed that GPDM abundance and activity were significantly elevated in prostate cancer cells compared to normal cells. Furthermore, both the glycolytic capacity and glycerophosphate-dependent ROS production were increased in the tumor cells. These data demonstrate that GPDM is involved in maintaining a high rate of glycolysis and represents an important site of electron leakage leading to ROS production not only in prostate cancer cells but also in pancreatic cancer cells as given by our own results. This hypothesis is supported by our observation of an up-regulated expression of the mitochondrial precursor of gluthathione reductase (GSHR) in IL-1-treated Colo357 cells. GSHR adds to the list of enzymes involved in oxidative stress and whose expression is up-regulated in different tumor cells (39). Moreover, oxidative stress has been shown to induce chromosomal instability in pancreatic cells derived from patients with chronic pancreatitis and pancreatic adenocarcinoma. These results might give further insight into the linkage of tumor-associated chronic inflammation and pancreatic cancer (40).

Three further proteins co-chromatographing with GSHR were identified as UTP-glucose-1-phosphate uridyltransferase 2 (UGPA), mitochondrial precursor of ATP synthase unit alpha (ATP6) and nicotinamide phosphoribosyltransferase (NAMPT). We assume that the intensity of the corresponding spot is caused by GSHR, NAMPT and ATP6 rather than by UGPA because an increase in this enzyme involved in glycogenesis does not fit with metabolic stress regarding mobilization of energy reserves necessary for unlimited cell division. This assumption is supported by the finding of an up-regulated expression of ATP6 in liver metastases of colorectal cancer (41). Interestingly, Dowling *et al* observed a correlation between the increased abundance of ATP6 and the progression of cancer, since up-regulation of the enzyme contributed to the enhanced invasive potential of human breast cancer cells leading to a more aggressive phenotype (42).

All of the proteins mentioned before are more likely markers of the highly proliferative status of the tumor cells and do not provide the basis for new therapeutical concepts in cancer treatment, but two further proteins identified by our proteomic approach might be considered as putative target molecules for this challenging objective: these molecules are NAMPT and prostaglandin H_2 synthase 2 (PGHS-2). NAMPT is also known as visfatin or pre-B cell colony enhancing factor (PBEF). To our knowledge this report is the first one describing an IL-1-induced increase in the expression of NAMPT in a tumor model for human pancreatic cancer. We were able to demonstrate an enhanced expression of NAMPT at both, the mRNA and the protein level. The expression rate was nearly doubled within the first 24 h of incubation with the cytokine and then remained unaffected. It is well established that an inflammatory stimulus is implicated in the up-regulation of the *NAMPT* gene expression mediated by the transcription factors NF-κB and AP-1 (43).

Over-expression of NAMPT also fits into the picture of fast dividing tumor cells. It is the main enzyme for the regeneration of nicotinamide-D-ribonucleotide by transferring nicotinamide to 5'-phospho-α-D-ribose 1-diphosphate (44). Recently, NAMPT was described as visfatin belonging to the group of

adipocytokines. These proteins are mainly adipocyte-derived cytokines affecting immune and inflammatory functions. Moschen *et al* demonstrated an activation of human leukocytes by visfatin resulting in the production of pro-inflammatory mediators including IL-1, TNF-α, and IL-6 (45). From these observations the authors postulated that visfatin can be considered as a new pro-inflammatory adipocytokine.

The over-expression of NAMPT found in Colo357 supports the idea of an association of chronic inflammation with the development of pancreatic malignancy. Moreover, Van der Veer *et al* identified a relationship between aging of human vascular smooth muscle cells (SMCs) and visfatin (46). Replicative senescence of SMCs was preceded by a marked decline in the expression and activity of NAMPT. In contrast, introduction of the *NAMPT* gene into aging human SMCs delayed senescence and substantially lengthened cell life-span, together with enhanced resistance to oxidative stress. NAMPT over-expression also reduced an age-related increase in p53 expression and increased the rate of p53 degradation. This feature of NAMPT may raise the tumorigenicity of cells bearing higher amounts of this enzyme like Colo357. An up-regulated expression of NAMPT was also described for primary colorectal cancer and breast cancer (47,48). Since Folgueira *et al* detected a higher resistance of NAMPT over-expressing tumor cells to chemotherapy (47), we suggest that IL-1-induced NAMPT over-expression observed in Colo357 might also contribute to the chemoresistance of pancreatic carcinoma. It has been shown previously that NAMPT mediates the stabilization of chromatin by poly-ADP-ribosylation resulting in a higher resistance to DNA-damaging substances not only in healthy but also in cancer cells (49). In this context inhibition of NAMPT appears to represent a striking tool in anti-tumor therapies. At present two pharmacological inhibitors are under investigation: FK866 and CHS-828 (50). In clinical studies with patients harboring different highly advanced malignancies resistant to common therapies the pharmacokinetics and the action of the inhibitors showed large variations among patients. There was no regression of tumor mass at all but tumor progression was inhibited in response to the inhibitors in some cases (51,52)

In four of nine up-regulated spots identified by our proteome analysis we found IL-1-dependent induction of PGHS-2 in Colo357. An enhanced expression of the enzyme has been reported in different tumor entities besides pancreatic carcinoma (53,54). In Colo357 we could demonstrate an elevated PGHS-2 synthesis at both the mRNA and the protein level induced by IL-1. The amount of the enzyme-specific mRNA reached a plateau after 24 h of IL-1 treatment and decreased thereafter. This indicates the involvement of some products of the enzyme in regulating mRNA expression. In general, PGHS-2 is an inducible form of cyclooxygenases. Cyclooxygenases catalyze the production of prostaglandin H_2 (PGH_2), which is the basic compound for a great panel of different prostanoids (55). Because of the variety of PGHS-2 products the characteristic expression profile of its corresponding mRNA might be involved in inhibiting the flushing of the cells and their microenvironment by mediators of inflammation and pain. PGHS-2 gene expression is regulated at the levels of mRNA stability and translation efficiency governed by multiple regulatory elements located in its 3'-untranslated region. Specific microRNA, RNA-binding proteins as well as alternative polyadenylation affect PGHS-2 mRNA stabilization thus regulating gene expression (56). Under normal conditions, PGHS-2 mRNA is rapidly degraded in processing bodies (57). These P bodies are conserved structures present in a great variety of eukaryotic cells and have been found as playing a crucial role in mRNA decay and storage (58). Our own results are in good agreement with these findings because we observed a rapid decay of the IL-1-induced PGHS-2 mRNA expression after reaching a maximum. Consequently, PGHS-2 protein amount in Colo357 peaked with a delay of 24 h and declined subsequently. The association between carcinogenesis and elevated levels of PGE_2, one of the secondary products of PGH_2, is well established in colorectal adenoma in familial adenomatosis polyposis patients. The resulting ratios were correlated with the size of the adenoma suggesting a critical role of prostanoids in tumor progression (59). Moreover, Fukuda *et al* demonstrated that PGE_2 exposure induced the expression of vascular endothelial growth factor (VEGF) mRNA in human colon carcinoma cells (60). In a breast cancer study the expression pattern of PGHS-2 was found as being tightly correlated with lung-metastatic activity in the tumor patients (61). Gupta *et al* could show that the tumor-specific expression of epidermal growth factor receptor ligand epiregulin, PGHS-2, and matrix metalloproteinases 1 and 2 collectively facilitate the assembly of new tumor blood vessels and the release of tumor cells into the circulation (62). Taken together, these results emphasize PGE_2 as being one of the most important mediators of PGHS-2-associated tumor invasiveness. Okami *et al* demonstrated the effectiveness of the PGHS-2-specific inhibitor JTE-522 in reducing the invasive potential of pancreatic cancer (63). Based on these findings several phase II clinical trials on the treatment of patients with advanced pancreatic carcinoma had been initiated using selective PGHS-2 inhibitors in combination with certain cytostatics. From these studies it became obvious that PGHS-2 inhibitors alone may not be sufficient to sensitize pancreatic cancer to the effects of conventional cytotoxic therapy (64,65).

On the other hand, selective PGHS-2 inhibitors have come under scrutiny because of reports suggesting an increased cardiovascular risk associated with their use (66). The hitherto used high therapeutic concentrations of these drugs may contribute to a prothrombotic state in patients with higher risk for serious cardiovascular events.

Taken together, all these results clearly demonstrate that there is a strong medical need for the development of new concepts how such deadly biological activities working in pancreatic cancer may be therapeutically targeted with combinations of low-dose chemopreventive drugs. In this context and based on the presented data for Colo357 cells, inhibitors of NAMPT and PGHS-2 are promising candidates for a combinatory blockage of two critical enzymes over-expressed in pancreatic cancer cells. It has been shown previously that the PGHS-2-specific inhibitor celecoxib and the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) act synergistically in inhibiting cellular growth and inducing apoptosis in a model of prostate carcinoma (67).

On the basis of the presented work we propose a combinatorial administration of NAMPT and PGHS-2 inhibitors

as an adjuvant therapy in pancreatic cancer. It has to be seen primarily as an attempt to supplement the hitherto existing conventional methods to fight the poor outcome of the disease. Moreover, the aim is to improve the existing therapeutical networks. These novel approaches might disturb the concert between inflammation and malignant transformation of pancreatic cells leading to a better prognosis and prolonged overall survival of high-risk patients with pancreatic neoplasia.

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