Enhanced expression of 14-3-3sigma in pancreatic cancer and its role in cell cycle regulation and apoptosis

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14-3-3sigma belongs to the 14-3-3 family of proteins, which are involved in the modulation of diverse signal transduction pathways. Loss of 14-3-3sigma expression has been observed in a number of human cancers, suggesting that it may have a role as a tumor suppressor gene. The aim of the study was to investigate the expression and the functional role of 14-3-3sigma in pancreatic ductal adenocarcinoma (PDAC). Expression of 14-3-3sigma was analyzed using laser capture microdissection (LCM), quantitative real-time-PCR (QRT-PCR), DNA arrays, immunohistochemistry and western blot analysis. The role of 14-3-3sigma in apoptosis and cell cycle regulation was evaluated by western blotting, immunoprecipitation and FACS analysis. By QRT-PCR, 14-3-3sigma mRNA levels were 54-fold increased in pancreatic adenocarcinoma in comparison with normal pancreatic samples and localized in pancreatic cancer cells as determined by LCM. In pancreatic cancer cells, the degree of 14-3-3sigma expression was not decisive for the maintenance of G2/M cell cycle checkpoint or induction of apoptosis. Responses to radiation or apoptosis-inducing agents were neither accompanied by a significant 14-3-3sigma accumulation nor by a change in association of 14-3-3sigma with cdc2, bad and bax. In conclusion, the marked over-expression of 14-3-3sigma in PDAC together with multiple known genetic and epigenetic alterations of potential 14-3-3sigma interacting partners suggests an important role of aberrant 14-3-3sigma downstream signaling in pancreatic cancer.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth to fifth leading cause of cancer-related mortality in the western world (1,2). It is a devastating disease with a poor prognosis, an overall 5-year survival rate of < 1%, and a median survival time after diagnosis of ~5–6 months. At the time of diagnosis between 75 and 85% of PDAC patients have unresectable tumors, and conventional therapies are virtually ineffective (1–4). An improved understanding of the molecular characteristics of PDAC is the only means of providing new markers for early diagnosis and identifying potential targets for therapeutic intervention. Therefore, in recent decades many laboratories have focused on understanding the molecular alterations that occur in PDAC.

During the process of malignant transformation, pancreatic cancer cells acquire resistance to apoptosis mediated by conventional chemotherapeutic agents or by apoptosis-inducing receptors such as Fas or TNF-R (5,6). Resistance to apoptosis is mediated through a number of mechanisms, including p53 and K-ras mutations, aberrant expression of genes that induce or prevent apoptosis, such as bax and bcl-2, respectively (7), and excessive activation of mitogenic pathways (8). Another hallmark of malignant transformation is deregulated cell cycle control. Defects in cell cycle checkpoints can be the result of gene mutations, chromosome damage and aneuploidy, all of which result in permanent alterations of the genome (9). The checkpoint mechanisms that normally regulate cell cycle progression are frequently disrupted in tumor cells (10,11).

14-3-3 proteins constitute a large family of acidic soluble proteins that are expressed in virtually every organism studied so far, ranging from mammals to yeast (12–17). In humans, there are seven distinct 14-3-3 genes, denoted β, γ, ε, η, δ, τ and ζ (as well as a number of potential pseudogenes). Despite this genetic diversity, 14-3-3 proteins exhibit a remarkable degree of sequence homology and conservation between species (18–20). 14-3-3 proteins bind to discrete phosphoserine-containing motifs present in many signaling molecules. However, they are also capable of interacting with unphosphorylated ligands (21,22). At least 100 different binding partners for 14-3-3 proteins have been identified, many of which participate in the modulation of various cellular processes such as cell cycle progression, apoptosis, signal transduction, stress response, cytoskeleton organization and malignant transformation (23–27).

The 14-3-3sigma gene (also called stratifin) was originally characterized as the human mammary epithelial-specific marker, HME-J (28), and is expressed in keratinocytes (29) and epithelial cells (30). 14-3-3sigma is up-regulated through a p53-dependent mechanism following DNA damage (31), and sequesters cyclin B1/CDC2 complexes in the cytoplasm during G2 arrest. Its absence allows cyclin B1/CDC2 complexes to enter the nucleus, causing mitotic catastrophe (32,33). 14-3-3sigma has also been shown to specifically interact with CDK2, CDC2 and CDC4 and to inhibit CDK activities, thereby blocking cell cycle progression, thus defining it as a new class of CKI (33). Besides its G2/M checkpoint functions, 14-3-3sigma has an anti-apoptotic role through its inhibitory interactions with the pro-apoptotic proteins Bad and Bax. Thus, 14-3-3sigma sequesters phosphorylated Bad and Bax in the cytoplasm, thereby preventing Bad from associating with bcl-XL and Bax from translocating to the mitochondria (34,35).

The involvement of 14-3-3sigma in human cancer has been established in studies of breast cancer in which...
methylation-dependent silencing of the gene was observed in the majority of cases (36), and loss of 14-3-3-sigma has been shown to occur early in the neoplastic development of breast epithelium (37). Silencing of 14-3-3-sigma expression has been reported in 43% of primary gastric adenocarcinomas (38) and 89% of hepatocellular carcinomas (39), as well as in certain squamous cell carcinomas (40,41). In contrast, 14-3-3-sigma is reportedly up-regulated in lung cancer (42) and in head and neck squamous cell carcinoma (43). Previously, using DNA array technology, we and others have demonstrated an increase of 14-3-3-sigma mRNA expression in PDAC in comparison with the normal pancreas (44–46). In the present study, we investigated 14-3-3-sigma localization, expression and function in PDAC in order to assess its potential role in this disease.

Materials and methods

Patients and tissue collection

Human PDAC tissue samples were obtained from 52 patients (25 female, 27 male; median age 65 years; range 28–82) who underwent pancreatic resection at the University Hospital of Berne (Switzerland) and the University Hospital of Heidelberg (Germany). Normal human pancreatic tissue samples were obtained through an organ donor program from 24 previously healthy individuals (11 females, 13 males; median age 45 years; range 20–74). Freshly removed tissue samples were immediately fixed in paraformaldehyde solution for 12–24 h and paraffin-embedded for immunohistochemical analysis. Concomitantly, tissue samples for RNA extraction were immediately snap-frozen in liquid nitrogen in the operating room upon surgical removal and maintained at −80°C until use. The human subject committees of the Universities of Bern and Heidelberg and of Dartmouth Medical School approved the studies.

dNA array

The HG-U95Av2 array was obtained from Affymetrix (Santa Clara, CA). It contains about 10 000 full-length human genes from the Unigene database. Poly (A) + RNA isolation, cDNA synthesis, and cRNA in vitro transcription was carried out as described previously (44,47). The in vitro transcription product was purified and fragmented as described (44,47). Hybridization of the fragmented in vitro transcription products to oligonucleotide arrays was performed as suggested by the manufacturer (Affymetrix).

Light cycler quantitative real-time-PCR (QRT-PCR)

All reagents and equipment for mRNA/cDNA preparation were purchased from Roche Applied Science (Mannheim, Germany). mRNA was prepared by automated isolation using MagNA Pure LC instrument and isolation kits I (for cells) and II (for tissues). cDNA was prepared using the first strand cDNA Synthesis Kit for RT-PCR according to the manufacturer’s instructions. QRT-PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit as described previously (48). The number of 14-3-3-sigma-specific transcripts was normalized to housekeeping genes (cyclophilin B and hypoxanthine guanine phosphoribosyltransferase; HPRT). All primers were obtained from Search-LC (Heidelberg, Germany).

Laser capture microdissection

Tissue samples were embedded in OCT (Sakura Finetek, Torrance, CA) by freezing the blocks in an acetone bath within liquid nitrogen. Tissues were then stored at −80°C until use. Tissue sections (6–8 μm thick) were prepared using a Reichard-Jung 1800 cryostat. The sections were attached to glass slides and stored at −80°C as described previously (48). Tissues were then dehydrated in 100% high-grade ethanol. The sections were then incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Then, the sections were incubated for 30 min at room temperature with 10% normal goat serum. After washing in 95% ethanol, sections were incubated for 30 min at room temperature with 1% non-immune horse serum (Dako, Glostrup, Denmark). Slides were washed twice in PBS and twice at 60°C in 0.1 × SSC, 2% SDS, and subsequently exposed at −80°C to Kodak BioMax films with Kodak intensifying screens for the appropriate time.

Immunohistochemistry

Immunohistochemical analysis was performed using the streptavidin–peroxidase technique and DAKO EnVision + System, Peroxidase (Dako Cytomation GmbH, Hamburg, Germany) according to the manufacturer’s protocol. Briefly, consecutive 3–5 μm paraffin-embedded tissue sections were deparaffinized and dehydrated. Slides were incubated for 10 min in 0.3% hydrogen peroxide to block endogenous peroxidase activity. Then, the sections were incubated for 30 min at room temperature with 10% normal goat serum prior to overnight incubation at 4°C with mouse monoclonal anti-14-3-3-sigma (NeoMarkers, Westinghouse, CA) diluted in PBS (2 μg/ml). Secondary goat anti-mouse peroxidase-labeled polymer was used. Slides were counterstained with Mayer’s hematoxylin. To ensure specificity of the primary antibodies, consecutive tissue sections were incubated in the absence of the primary antibody. In these cases, no immunostaining was detected. In addition, skin sections were analyzed as positive controls.

Immunofluorescence

Paraffin-embedded tissue sections were deparaffinized and dehydrated. Then, sections were incubated for 30 min at room temperature with 10% normal goat serum prior to overnight incubation at 4°C with a mouse monoclonal anti-14-3-3-sigma antibody (NeoMarkers) diluted in PBS (2 μg/ml). Secondary goat anti-mouse Cy3 labeled antibodies were used. Slides were counterstained with DAPI.

Cell culture

ASPC-1, BaPc-3, Capan-1, COLO-357, Mia PaCa-2, PANC-1, T3M4 and Hela cells were grown in RPMI media. HCT 116 cells were grown in McCoy’s media. Cells were maintained at 37°C in humidified air with 5% CO2. Media were supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS. For induction of apoptosis, cells were exposed to actinomycin D (Sigma-Aldrich Chemie, Taufkirchen, Germany) at a concentration of 0.5 μg/ml. Irradiation was performed using a 137Cs γ-iradiator at 7.5 Gy/min for 2 min.

Mutation analysis

DNA was extracted from pancreatic cancer cell lines and skin as a positive control using the DNA extraction kit Qiagen (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s protocol. A 1.2-kb PCR product, encompassing the entire 14-3-3-sigma coding sequence, was generated by using two primers: 5’-GTCTGTTCCAGAGCCATG-3’ (sense) and 5’-GTCGCGTGTTCCAGAGCCATG-3’ (antisense). The PCR contained 1 μg of DNA, PCR Master Mix, Promega (Promega GmbH, Mannheim, Germany) (25 U/ml TaqDNA Polymerase, 200 μM each: dATP, dGTP, dCTP, dTTP, 1.5 mM MgCl2) in a 100 μl reaction volume. The PCR conditions were as follows: an initial denaturation step for 5 min at 94°C and then 30 s at 94°C, 60 s at 58°C.

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and 2 min at 72°C for 34 cycles followed by an elongation step for 8 min at 72°C. PCR products were resolved by electrophoresis in a 1% agarose gel pre-stained with ethidium bromide. The rest of the PCR product was purified and sent for sequencing by Qiagen (Qiagen GmbH).

**Antibodies**

The following primary antibodies were used: mouse anti-14-3-3-sigma Ab-1 (clone 1433501) (NeoMarkers), rabbit polyclonal anti-Bad (H-168), agarsase conjugate mouse monoclonal anti-Bax (C-7), rabbit polyclonal anti-Bax (N-20), agarsase conjugate rabbit polyclonal anti-Bax (N-20), mouse monoclonal anti-cdc2 p34(17), mouse monoclonal anti-cyclinB1 (GSN1), goat polyclonal anti-cdc25c (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-pCdc2 (Tyr 15) 9111s (Cell Signaling Technology, Beverly Hills, CA).

**Western blot**

Cultured pancreatic cancer cells were washed in ice-cold PBS, scraped, centrifuged briefly and lysed in 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% SDS) supplemented with the Complete-TM mixture of protease inhibitors (Roche Diagnostics). Cells were centrifuged (14 000 r.p.m., 30 min at 4°C), the supernatants were collected and the protein concentration was measured with the BCA protein assay (Pierce Chemical, Rockford, IL). Protein (15 ug/sample) was diluted in sample buffer (250 mM Tris-HCl, 4% SDS, 10% glycerol, 0.066% bromophenol blue and 2% β-mercaptoethanol), boiled for 5 min, cooled on ice for 5 min, and size-fractionated on 12% SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes at 100 V for 90 min. Subsequently, membranes were incubated for 1 h in a blocking solution (5% non-fat milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20), followed by incubation with the indicated primary antibodies in blocking solution overnight at 4°C. Membranes were then washed with the blocking solution and incubated with the corresponding horseradish peroxidase conjugated secondary antibodies for 60 min at room temperature. Antibody detection was performed with the enhanced chemoluminescence western blot detection system (Amersham Life Science, Amersham, UK). For loading and transfer control, anti-ERK2 rabbit polyclonal antibodies were utilized (Santa Cruz Biotechnology).

**Immunoprecipitation**

For immunoprecipitation (IP), cells were washed twice with ice-cold PBS and lysed in buffer containing 50 mM Tris pH 7.4, 250 mM NaCl, 1% Triton X-100 supplemented with the Complete-TM mixture of protease inhibitors (Roche Diagnostics), 2 mM PMSF, 50 mM NaF, 1 mM sodium orthovanadate and 0.5 mM sodium para phosphate for at least 30 min on ice. Following centrifugation the supernatant was incubated overnight at 4°C with the indicated antibody, and the antibody-bound complexes were precipitated for 60 min with 10 µl of protein G-Sepharose (Santa Cruz Biotechnology). The immunoprecipitates were washed three times with lysis buffer without additional steps, and then eluted with 80 µl of 2 x SDS sample buffer for analysis by SDS-polyacrylamide gel electrophoresis and immunoblotting.

**FACS analysis**

**Cell cycle analysis.** Both adherent and floating cells were collected for analysis. Cell cycle analysis was performed using a hypotonic fluorochrome solution (propidium iodide 50 µg/ml, sodium citrate 0.1%, Triton X-100 0.1%). Flow cytometry was performed with a FACS instrument (Becton Dickinson Biosciences, Erembodegem, Belgium).

**Detection of apoptosis.** Detection of apoptosis was carried out by the Annexin-V-Fluos kit (Roche Diagnostics). Both adherent and floating cells were collected for analysis. Cells were collected by centrifugation at 200 g for 5 min. Subsequently the cell pellet was re-suspended in 100 µl Annexin-V-Fluos in a HEPES buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM sodium chloride, 5 mM calcium chloride) containing propidium iodide and incubated for 10-15 min. Subsequently, flow cytometry was performed with a FACS instrument (Becton Dickinson Biosciences, Erembodegem, Belgium).

**Results**

**Expression and genomic analysis of 14-3-3-sigma**

We first screened the mRNA expression of 14-3-3 family members in pancreatic cancer, chronic pancreatitis and the normal pancreas utilizing DNA arrays. This analysis revealed that 14-3-3-sigma is the most prominently over-expressed isoform of the 14-3-3 family in pancreatic cancer compared with normal pancreatic tissues (Table I). Thus, 14-3-3-sigma mRNA expression levels were increased 11.6- and 3.5-fold in pancreatic cancer compared with chronic pancreatitis and the normal pancreas, respectively. Furthermore, in metastatic lesions, 14-3-3-sigma levels were increased 19.2- and 1.6-fold in comparison with normal pancreatic and primary cancer samples, respectively. In contrast, the other 14-3-3 isoforms displayed different patterns. 14-3-3-sepsilon and tau mRNA levels were down regulated in pancreatic cancer in comparison with normal pancreatic tissues, whereas 14-3-3-beta, eta and zeta expression was slightly increased in pancreatic cancer. These findings are in agreement with previous DNA array results in pancreatic cancer that specifically identified 14-3-3-sigma (and not the other 14-3-3 isoforms) as an over-expressed gene in this disease (44-46).

To more accurately quantify this over-expression, QRT-PCR was carried out using whole tissue RNA from normal (n = 24) and PDAC (n = 52) tissues. This analysis revealed that there was a 54 ± 9.0-fold (mean ± SEM) increase in 14-3-3-sigma levels in PDAC as compared with the normal pancreas (Figure 1A). Moreover, 39 of 52 PDAC samples (75%) exhibited 14-3-3-sigma mRNA levels that exceeded the highest 14-3-3-sigma levels in normal tissues. Next, RNA was extracted from laser-captured ductal cells obtained from three normal pancreas samples, five chronic pancreatitis samples, and six PDAC samples. In addition, RNA was prepared from three samples of total, non-microdissected, normal pancreatic tissues. After reverse transcription, mRNA expression levels were assessed by QRT-PCR analysis. The microdissected normal ducts and the ductal cells in chronic pancreatitis exhibited moderate levels of 14-3-3-sigma, whereas total pancreas samples (containing mostly acinar cells) displayed relatively low levels of 14-3-3-sigma (Figure 1B). Moreover, the microdissected cancer cells showed a 3 ± 0.9-fold increase in 14-3-3-sigma mRNA levels when compared with the microdissected non-neoplastic ducts, and a 40 ± 12-fold increase when compared with total pancreatic tissue samples (Figure 1B). Southern blot analysis revealed no obvious difference in the number of gene copies in pancreatic cancer samples in comparison with normal pancreatic tissues and no gross genomic rearrangement (Figure 1C).

Immunohistochemical localization of 14-3-3-sigma was carried out next using 10 normal pancreatic tissues, 20 primary and 10 metastatic PDAC samples. 14-3-3-sigma immunoreactivity was absent in all 10 normal pancreatic tissues and was not detectable in either the acinar, ductal or islet cells (Figure 2A and B). In contrast, in all 20 primary PDAC samples, strong cytoplasmic 14-3-3-sigma immunostaining was present in the cancer cells (Figure 2C and D). Similarly, strong 14-3-3-sigma immunoreactivity was observed in the cytoplasm of the cancer

**Table 1. Expression of different 14-3-3 isoforms in the normal pancreas (No), chronic pancreatitis (CP), primary pancreatic cancer (CA) and pancreatic cancer metastasis (Mx) as determined by DNA array analysis**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Normal vs No</th>
<th>PDAC vs CP</th>
<th>PDAC vs Mx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta</td>
<td>2.79</td>
<td>1.82</td>
<td>3.39</td>
</tr>
<tr>
<td>Epsilon</td>
<td>2.32</td>
<td>2.00</td>
<td>1.78</td>
</tr>
<tr>
<td>Eta</td>
<td>1.70</td>
<td>1.22</td>
<td>1.69</td>
</tr>
<tr>
<td>Sigma</td>
<td>11.61</td>
<td>3.45</td>
<td>19.23</td>
</tr>
<tr>
<td>Tau</td>
<td>-1.61</td>
<td>-1.69</td>
<td>-1.70</td>
</tr>
<tr>
<td>Zeta</td>
<td>2.06</td>
<td>1.54</td>
<td>2.05</td>
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the specificity of the antibody, skin sections were also analyzed. As described previously (29), positive cytoplasmic staining of keratinocytes could be detected (Figure 3C). Overexpression of 14-3-3sigma at the protein level was also confirmed by western blot analysis of normal tissues and pancreatic cancer samples (Figure 1D).

The expression level of 14-3-3sigma was examined next in eight pancreatic cancer cell lines. BxPc-3, Colo-357 and T3M4 cells exhibited relatively high levels of 14-3-3sigma mRNA (Figure 4A) and protein (Figure 4B). Aspc-1 and Capan-1 cells exhibited moderate levels of 14-3-3sigma mRNA (Figure 4A) and protein (Figure 4B). Panc-1 cells exhibited low levels of 14-3-3sigma mRNA (Figure 4A) and protein (Figure 4B), and Mia-PaCa-2 cells exhibited very low levels of 14-3-3sigma mRNA (Figure 4A) and no detectable protein levels (Figure 4B). It has been shown previously that low expression levels in these cells are due to promoter hypermethylation (46,50). In order to determine whether there were mutations of the 14-3-3sigma coding sequence in pancreatic cancer cells, full-length DNA sequencing of ASPC-1, Mia-Paca2, T3M4 and skin (as a positive control) was performed. No mutation was detected in the coding sequence in any of the samples (data not shown).

14-3-3sigma and G2 cell cycle checkpoint in pancreatic cancer

Following DNA damage, 14-3-3sigma is up-regulated both in human colon carcinoma cells and in mouse ES cells by a p53- and BRCA1-dependent mechanism, thereby blocking G2/M progression (31,32,51). One of the mechanisms by which this G2/M block is mediated is the subcellular translocation of Cdc2. During the G2 phase, the Cdc2/cyclin B complex is kept inactive by phosphorylation of tyrosine 15 and threonine 14 residues of Cdc2. At the onset of mitosis, both of these residues are dephosphorylated (activated) by the phosphatases Cdc25c and Cdc2. Subsequently, the Cdc2/cyclin B complex translocates to the nucleus and initiates mitosis (52,53). 14-3-3sigma binds to Cdc2 and blocks entry into mitosis by anchoring the Cdc2/cyclin B1 complex in the cytoplasm, resulting in G2/M block (31).

To elucidate the role of 14-3-3sigma in mediating the effects of DNA-damaging agents in pancreatic cancer cell lines, we performed cell cycle analysis in cells with high and low levels of 14-3-3sigma. We also examined the expression levels of Cdc2, cyclin B1, Cdc25c and p21. The cell lines examined were Colo-357 (p53 wild-type and high levels of 14-3-3sigma), T3M4 (p53 mutated and high levels of 14-3-3sigma), and Mia-Paca-2 (p53 mutated and low levels of 14-3-3sigma). Cells were exposed to gamma irradiation (15 Gy) followed by FACS analysis, QRT-PCR and western blot analysis at 0, 1 and 48 h after treatment. Based on the FACS analysis, following irradiation Colo-357 and Mia-Paca-2 were blocked in the G2/M-phase of the cell cycle, while T3M4 showed S-phase accumulation (Figure 5). In T3M4 cells, QRT-PCR analysis revealed transiently increased levels of 14-3-3sigma mRNA after irradiation in comparison with its basal levels, while in Mia-Paca2 and Colo-357 cells no change in the basal 14-3-3sigma mRNA levels was observed (data not shown). Western blot analysis showed only a significant increase of the basal levels of 14-3-3sigma protein in T3M4 cell lines after 1 h of irradiation, while no significant change was observed in Colo-357 and Mia-Paca2 cells (Figure 6). In addition, following irradiation, there was a slight increase in
p21 protein levels in Colo-357 pancreatic cancer cells, but not in the other tested cell lines (Figure 6).

Since no correlation between G2 block and 14-3-3sigma levels could be established, we next analyzed the availability of required protein partners. Western blot analysis for Cdc2 and Cdc25c showed that after exposure to radiation, both phosphorylated and non-phosphorylated forms of Cdc2 accumulated in T3M4 and Mia-Paca2 cells, while Colo-357 showed no difference in the basal levels of Cdc2 (Figure 6). Cdc25c was not expressed in the examined pancreatic cancer cell lines (Figure 4B). Taken together, these results might suggest that in pancreatic cancer 14-3-3sigma is not crucial for maintenance of G2 checkpoint integrity.

14-3-3sigma and induction of apoptosis in pancreatic cancer

14-3-3sigma is known to exert anti-apoptotic effects by interacting with and inhibiting the actions of bad and bax pro-apoptotic proteins (34,35). Bad induces apoptosis by binding to and inhibiting the anti-apoptotic effects of bcl-xL and bcl-2. 14-3-3sigma sequesters phosphorylated bad in the cytosol, thereby preventing it from interacting with bcl-xL (54,58). 14-3-3sigma also binds to and sequesters bax in the

Fig. 2. Localization of 14-3-3sigma in pancreatic tissues. Immunohistochemistry was carried out using a specific 14-3-3sigma antibody as described in the Materials and Methods section; (A and B) normal pancreas; (C and D) pancreatic cancer; (E and F) liver metastases of pancreatic cancer. Note the strong 14-3-3sigma immunoreactivity in the metastatic pancreatic cancer cells in comparison with the adjacent normal liver, as seen in insert (E).
cytoplasm, thereby preventing the translocation of bax to the mitochondria and blocking the cytoplasmic release of cytochrome c (34,35).

To induce apoptosis, Colo-357, Mia-Paca-2 and T3M4 cells were treated with 500 ng/ml actinomycin D (59) for 12 h and then subjected to analysis by FACS, western blotting and immunoprecipitation. In 14-3-3-sigma-high Colo357 cells, very limited apoptosis occurred (Figure 7), without obvious change of 14-3-3-sigma protein levels (Figure 8). However, both 14-3-3-sigma-high T3M4 and 14-3-3-sigma-low MiaPaca2 showed prominent induction of apoptosis (Figure 7), with no other significant changes in cell cycle distribution for T3M4 cells and slight G1 accumulation for Mia-Paca2 cells (data not shown). In T3M4 cells, 14-3-3-sigma protein decreased slightly after treatment with actinomycin D, but Mia-Paca2 did not display detectable levels of 14-3-3-sigma prior to or after actinomycin D treatment (Figure 8). The reason for and the functional consequences of reduced levels of 14-3-3-sigma in T3M4 cells following drug exposure are currently not known.

Analysis of expression of proteins known to be targeted by 14-3-3-sigma during its anti-apoptotic action (Cdc2, cyclin B1, bad and bax) revealed that after actinomycin D treatment, the phosphorylated Cdc2 protein was significantly down-regulated in MiaPaca2 and T3M4 cells and to a lesser extent in Colo-357 cells (Figure 8). Significant down-regulation of cyclin B1 protein was observed in Mia-Paca-2 and T3M4 cells. Cleavage of bax protein after treatment with actinomycin D was observed in Colo-357 and T3M4 cells (Figure 8), but not in Mia-Paca-2 cells. Levels of bad protein remained unchanged (Figure 8).

To investigate the molecular mechanism of 14-3-3-sigma interaction with its expected partners in pancreatic cancer cell lines, we carried out immunoprecipitation experiments. Colo-357, T3M4 and Mia-Paca-2 cell lines, treated with actinomycin D, were analyzed for complex formation between 14-3-3-sigma and Cdc2, cyclin-B1, bad and bax. In Colo-357 and T3M4 cells, a spontaneous complex formation of 14-3-3-sigma with Cdc2 was observed, with or without actinomycin D treatment (Figure 9A). No complex formation was detected between 14-3-3-sigma and bad or between 14-3-3-sigma and bax. Reversed immunoprecipitation with bad, bax and Cdc2 confirmed these results (Figure 9B and C). In Mia-Paca-2 cells, with undetectable basal and induced levels of 14-3-3-sigma, no complexes were detected.
Next, to exclude possible false negative results, co-IP experiments were carried out in HCT116 cells, which express wild-type p53 and 14-3-3sigma and for whom an interaction between 14-3-3sigma and its partners, bad, bax and Cdc2 has been reported (32,35,60). As reported previously, there was a complex formation in HCT116 cells for 14-3-3sigma and its partners including bax, bad and Cdc2 (Figure 10).

In conclusion, in pancreatic cancer cells, 14-3-3sigma levels do not seem to correlate with the outcome of apoptosis induction, possibly due to improper complex formation with protein targets.

**Discussion**

Deregulation of 14-3-3sigma expression has been observed in a wide variety of human cancers, with both decreasing and increasing 14-3-3sigma levels being associated with development of malignancy. Loss of 14-3-3 sigma expression has been reported in primary gastric (38), hepatocellular (39) and breast (36) adenocarcinoma, and certain squamous cell carcinomas (40,41). Furthermore, 14-3-3sigma has been described as a potential marker for the non-cancerous state of epithelial cells (28,29). Also, lack of 14-3-3sigma in colorectal tumor cells was shown to sensitize them to chemotherapy-induced apoptosis. One of the major mechanisms of the 14-3-3sigma down-regulation seems to be methylation-dependent silencing (36). Recently, strategies aiming to correct 14-3-3sigma expression during chemotherapy have been considered to be of therapeutic interest (30). In contrast, 14-3-3sigma expression is up-regulated in head and neck squamous cell carcinoma and in chemoresistant pancreatic adenocarcinoma cells (61). Apparently, due to diversity of 14-3-3sigma functions, its role in cancerogenesis might be restricted to the particular type of tumor. To exert its effects, 14-3-3sigma recruits several interacting partners, suggesting that the functional status of downstream targets will at least partially determine its effects.

In the present study, we confirmed the up-regulation of 14-3-3sigma mRNA and protein in malignant cells of PDAC as compared with the normal pancreas. No mutation in the coding sequence of 14-3-3sigma was detected by sequencing analysis, indicating that cancer cell-associated 14-3-3sigma is potentially functional although other genetic and epigenetic alterations cannot be ruled out.

Since p53-controlled 14-3-3sigma expression was recognized as a key event in maintaining the G2 checkpoint after DNA damage, we expected that the pattern of responses to radiation would be determined by the individual p53 and 14-3-3sigma status in pancreatic cancer cells. Surprisingly, no such correlation could be seen: both Colo-357 (p53wt, 14-3-3sigma ++ ) and Mia-Paca2 (p53mutated, 14-3-3sigma +/-) entered and maintained G2 block in response to irradiation. Interestingly, transient up-regulation of already high 14-3-3sigma levels after DNA damage was only seen in S-phase arrested T3M4 cells (p53mutated, 14-3-3sigma ++ ), which express a mutated form of p53, indicating that pancreatic cancer cells possess alternative pathways for p53-independent 14-3-3sigma induction and 14-3-3sigma-independent G2 checkpoint maintenance. In addition, expression of 14-3-3sigma in several pancreatic cancer cell lines with mutated p53 phenotype strongly indicates that 14-3-3sigma expression in pancreatic cancer is controlled by an alternative p53-independent mechanism. It could be speculated that other p53 family members such as p63 or p73 (62) might be responsible for the irradiation induced increase in 14-3-3sigma levels in T3M4 cells and the high basal 14-3-3sigma levels in other pancreatic cancer cell lines.
**Fig. 6.** Effects of irradiation on the expression of 14-3-3sigma and its partners in pancreatic cancer cell lines. The indicated pancreatic cancer cells were exposed to 7.5 Gy/min for 2 min. One and 48 h later, proteins were extracted and western blot analysis for 14-3-3sigma, Cdc-2, phosphorylated Cdc-2 (p-cdc-2) and p21 was carried out as described in the Materials and Methods section. N, normal cells; R, irradiated cells; 1, 1 h; 48, 48 h.

**Fig. 7.** Effects of actinomycin D in pancreatic cancer cell lines. The indicated pancreatic cancer cell lines were treated with 0.5 μg/ml actinomycin D for 12 h. Then the cells were stained with Annexin-V and PI, and FACS analysis was carried out as described in the Materials and Methods section. The number in the upper left corner depicts the percentage of apoptotic cells, in the upper right corner the percentage of necrotic cells, and in the lower right corner the percentage of both apoptotic and necrotic cells.
Elaboration of experiments to study responses of pancreatic cancer cells to the apoptosis-inducing agent actinomycin D allowed us to elucidate molecular anomalies in 14-3-3sigma downstream signaling. Thus, we demonstrated for the first time that: (i) 14-3-3sigma was able to bind Cdc2 prior to DNA damage; (ii) 14-3-3sigma/Cdc2 complexes did not further accumulate after DNA damage and (iii) sequestered Cdc2 was present in the complex in its active tyr15-dephosphorylated form. Interestingly, although cyclin B1 was absent from constitutive 14-3-3sigma/Cdc2 complexes, treatment with actinomycin D led to the drastic down-regulation of this protein, a possible path to cell cycle arrest. The aspect of Cdc2-phosphorylation status is of particular interest, as the phosphatase Cdc25c was absent in all examined pancreatic cancer cells except BxPc-3 (Figure 4), which is in agreement with the observed reduced Cdc25c expression in pancreatic cancer tissues compared with normal controls (63). These findings indicate that Cdc25c, normally responsible for dephosphorylation and activation of Cdc2, might be replaced with other members of the Cdc25 family, such as Cdc25b (63), allowing initiation of mitosis.

Thus, although absence of 14-3-3sigma was shown previously to be associated with the inability to sustain the G2 checkpoint, leading to mitotic catastrophe, whereas 14-3-3sigma over-expression resulted in spontaneous ‘irradiation-like’ phenomenon, these consequences of 14-3-3sigma deregulation are apparently of no relevance in the case of pancreatic cancer: despite abundant amounts of 14-3-3sigma in tumor cells, its downstream partners, necessary for execution of this pathway, are missing or not presented in the right form.

Anti-apoptotic effects of 14-3-3sigma are ascribed to its interaction with and inhibition of the pro-apoptotic proteins bad and bax. Phosphorylated bad induces apoptosis by binding to and inhibiting the anti-apoptotic effects of Bcl-xL and Bcl-2. 14-3-3sigma sequesters phosphorylated bad in the cytosol, and then the 14-3-3sigma-bad complex is sequestered from mitochondrial localized Bcl-xL and thus inhibits bad-induced apoptosis (54–58). More recently it was reported that Cdc2 is capable of mediating apoptosis of cerebellar granule. Active Cdc2 catalyzes the phosphorylation of the BH3-only protein bad at a distinct site, serine 128, and thereby induces bad-mediated apoptosis in primary neurons by opposing growth factor inhibition of the apoptotic effect of bad. The phosphorylation of bad serine 128 inhibits the interaction of growth factor-induced serine 136-phosphorylated bad with 14-3-3 proteins (16). It was also reported that 14-3-3sigma could have an anti-apoptotic effect by direct interaction and sequestration of...
bax. With regard to the anti-apoptotic role of 14-3-3sigma in pancreatic cancer, our study showed that although both bad and bax proteins were present in cancer cells, neither a significant change of the protein level nor complexes with 14-3-3sigma were associated with actinomycin D-induced apoptosis. It seems that 14-3-3sigma does not interact directly with Bad and bax but could play an anti-apoptotic role by binding to and setting off the kinase activity of Cdc2, consequently prevent the induction of Cdc2-induced bad-mediated apoptosis.

In conclusion, accumulation of 14-3-3sigma in malignant cells is a hallmark of pancreatic cancer. However, due to multiple alterations of the interaction with downstream partners, 14-3-3sigma does not seem to carry out its major ascribed functions, such as the sustaining of a G2 checkpoint and anti-apoptotic action. Alternative pathways of 14-3-3sigma-signaling in pancreatic cancer and their pathologic consequences are the subject of further investigations.

References


Fig. 10. Effect of adriamycin on the association of 14-3-3sigma with its partners in HCT116 cells. HCT116 cells were treated with 0.5 μg/ml adriamycin for 48 h. Immunoprecipitation was carried out as described in the Materials and Methods section. (−) Untreated cells; (+) treated cells; (L) whole HCT116 cell lysate. Following 14-3-3sigma immunoblotting the membrane was exposed for 90 s (fourth panel). Longer exposure (overnight) revealed positive signals for bax and bad IP (fifth panel).
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