Overexpressed Decorin in Pancreatic Cancer: Potential Tumor Growth Inhibition and Attenuation of Chemotherapeutic Action

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ABSTRACT

Purpose: The aim of this study was to investigate the expression and significance of decorin in pancreatic cancer.

Experimental Design: Decorin expression in normal pancreas and excised tumors was examined by real-time quantitative PCR, Western blot analysis, and immunohistochemistry. Reverse transcription-PCR was used to analyze cultures of pancreatic cancer and stellate cells. Growth-inhibitory effects of decorin in vitro were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test, Western blot, and fluorescence-activated cell-sorting analysis.

Results: Pancreatic cancer was characterized by striking overexpression of decorin mRNA in tumor tissues (9-fold by real-time quantitative PCR; 44 patients versus 18 healthy donors; P < 0.01). Strong decorin immunostaining was observed in the extracellular matrix of pancreatic cancer tissue, whereas tumor cells were devoid of decorin. Double staining for anti-smooth muscle actin and decorin and reverse transcription-PCR analysis of primary cultures revealed pancreatic stellate cells as the putative source of decorin. Human recombinant decorin was able to suppress growth of pancreatic cancer cells in vitro through p21-mediated G₁-S block of the cell cycle. However, in contrast to the previously described chemotherapy-potentiating capacity of decorin, this proteoglycan attenuated the cytostatic action of carboplatin and gemcitabine toward pancreatic cancer cells. Overexpression of decorin in the tumor ECM, probably by myofibroblasts, has been postulated to represent a response of the host to invading cancer cells in an attempt to avert tumor

INTRODUCTION

Pancreatic cancer remains one of the leading causes of cancer deaths in the Western world. The 5-year survival for all stages of the disease remains <1% (1, 2). At resection, most of the patients present with late stages of the disease, which limits the role of surgery as a curative modality. Despite recent advances, adjuvant systemic therapies continue to be of limited value in pancreatic cancer patients (3).

The interaction of the peptic part of decorin with transforming growth factor (TGF)-β inhibits the binding of TGF-β to its receptor (20, 21). This is of particular importance because TGF-β production by tumor cells is implicated in the chemoresistance of malignant tumors. For example, in mammary cancer cell lines, development of chemoresistance was shown to be accompanied by rising TGF-β levels, and this process was reversed by the administration of the natural TGF-β antagonist decorin (22, 23).

Decorin expression is suppressed in breast, colon, gastric, hepatocellular, and ovarian cancer cells (5, 15, 22, 24–28). However, the mechanisms by which decorin transcription and translation are inhibited in these cancer cells are not yet clear. In the majority of tumors, the expression of decorin is already inhibited at the RNA level. Nevertheless, in ovarian cancer cell lines decorin transcripts are present, but there is no protein, indicating its rapid degradation by the ubiquitination pathway (15). Decorin has been proposed to be a tumor suppressor gene that is inactivated in cancer cells during malignant transformation, and overexpression of decorin in the tumor ECM, probably by myofibroblasts, has been postulated to represent a response of the host to invading cancer cells in an attempt to avert tumor
spread (15). The latter hypothesis is supported by the finding that low levels of small leucine-rich proteoglycans in breast tumors are associated with worse prognosis in lymph node-negative invasive breast carcinomas (29).

Recently, targeted decorin up-regulation has emerged as a feasible anticancer therapeutic modality. In one study, adenovirus-mediated transfer and expression of human decorin cDNA induced apoptosis of xenograft tumor cells in nude mice (30). This effect could be observed when the adenovirus vector encoding the decorin cDNA was injected intratumorally or i.v. Interestingly, injection of the decorin adenovirus vector into the tumor also resulted in growth inhibition of a distant contralateral tumor nodule. It could be shown that the effect of decorin was specific to tumor cells because neither apoptosis nor growth inhibition was observed in nontumor human cells such as hepatocytes, endothelial cells, or fibroblasts, despite p21 overexpression. Furthermore, in vitro studies demonstrated that the effects of carboplatin and decorin on ovarian cancer cells were synergetic (11).

Because decorin exerts tumor-inhibitory and chemotherapysynergistic activity, it represents an attractive prognostic marker as well as a target for potential therapeutic anticancer applications. Nothing is known about the role of decorin in pancreatic cancer, and therefore the aim of our study was to investigate decorin expression in human pancreatic cancer and gain an understanding of the involvement of decorin in the pathogenesis of this malignancy.

MATERIALS AND METHODS

Patients. All tissue specimens analyzed in this study were obtained according to institutional review board-approved procedures for consent. Human pancreatic cancer tissue samples were obtained from 44 patients (21 females and 23 males; median age, 65 years; age range, 28–82 years) who underwent pancreatic resection due to pancreatic cancer at the University Hospital of Berne (Berne, Switzerland) or Heidelberg (Heidelberg, Germany). Surgical procedures consisted of either a partial pancreaticoduodenectomy or another pancreatic resection. Eight patients presented with stage I disease, 5 patients presented with stage II disease, 24 patients presented with stage III disease, and 7 patients presented with stage IVa disease, according to the Union International Contre Cancer classification, 6th edition, 2002.

Normal human pancreatic tissue samples were obtained through an organ donor program from 18 previously healthy individuals (median age, 45 years; age range, 20–74 years) from whom other organs were taken for transplantation, and no recipients for the pancreas were present.

Tissue Sampling. Immediately after surgical removal, tissue samples were either snap-frozen in liquid nitrogen (for RNA and protein extraction) or fixed in 10% buffered formalin solution and embedded in paraffin 24 h later (for histological analysis).

Human Pancreatic Cancer Cell Lines. Pancreatic cancer cell lines MIA PaCa-2, Capan-1, Panc-1, BxPC-3 (American Type Culture Collection, Manassas, VA), T3M4, and Colo-357 (a gift of M. Korc; University of California, Irvine, CA) were cultured in complete RPMI 1640 containing 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), and 10% fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) at 37°C and 7% CO2.

Human Pancreatic Stellate Cells (PSCs). Human PSCs were cultivated in a 1:1 dilution of Ham’s F-12 nutrient medium and low-glucose DMEM (both from Invitrogen) supplemented with penicillin, streptomycin, amphotericin, and 20% fetal bovine serum.

Tumor Cell Growth Analysis. Cell proliferation was analyzed using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test for identification of living cells (Sigma-Aldrich Chemie, Deisenhofen, Germany). Briefly, 5 × 103 cells/100 μl were seeded in collagen-coated wells of flat-bottomed, 96-well plates (Sigmoidscreen; Sigma-Aldrich Chemie) and allowed to grow overnight. Afterward, triplicate cultures were either exposed or not exposed to human recombinant decorin at 100 μg/ml (EMP Genentech, Denzlingen, Germany) for 48 h. Ten μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) were added for 4 h, and formazan accumulation was measured as described previously (31).

To test for synergism between decorin and chemotherapeutic drugs, carboplatin (100 μg/ml; Bristol Laboratories, Princeton, NJ) or gemcitabine (12 ng/ml; Lilly Deutschland GmbH, Bad Homburg, Germany) was added to the pancreatic cancer cells (32), alone or simultaneously with decorin, and cells were incubated as described above.

Tumor Cell Cultures for Fluorescence-Activated Cell-Sorting and Western Blot Analyses. Cells (5 × 104 cells/well in complete medium) were seeded into collagen-coated, 24-well plates and allowed to grow overnight. After 24 subsequent hours of serum starvation, the cells were treated with decorin at 100 μg/ml for 24 h. For fluorescence-activated cell-sorting analysis, cells were harvested by trypsinization, washed, and kept in cold hypotonic propidium iodide buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide) until analysis. For Western blot analysis, cell monolayers were harvested using ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, and 1% SDS; supplemented with protease inhibitors (Roche); and then kept at −80°C until analysis.

Real-Time Quantitative PCR. All reagents and equipment for mRNA/cDNA preparation were purchased from Roche Applied Science (Mannheim, Germany). Messenger RNA was prepared by automated isolation using MagNA Pure LC instrument and isolation kits I (for cells) and II (for tissues). Complementary DNA was prepared using the 1st Strand cDNA Synthesis Kit for reverse transcription-PCR according to the manufacturer’s instructions. Real-time PCR was performed with the LightCycler FastStart DNA SYBR Green kit as described previously (33). All primers were obtained from Search-LC (Heidelberg, Germany). The number of specific transcripts was calculated from the standard curve and further normalized to the average expression of two housekeeping genes, cyclophilin B and hypoxanthine phosphoribosyltransferase. The data of two independent analyses for each sample and parameter were averaged and presented as adjusted transcripts/μl cDNA.

Western Blot Analysis. Western blot analysis was performed as described previously (34). Briefly, proteins were
extracted from normal human pancreatic tissues, pancreatic cancer tissues, and pancreatic cancer cell lines, and protein concentration was measured with the micro-BCA protein assay (Pierce, Rockford, IL). Twenty μg of cellular or tissue protein lysates were separated by 7.5% SDS-PAGE (decorin) or 12% SDS-PAGE (p21), transferred to nitrocellulose membrane, and blocked with 5% nonfat milk in TBS-T (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20) for 1 h. The membranes were incubated with the primary antibodies [anti-decorin polyclonal goat IgG (R&D Systems Europe Ltd., Abingdon, United Kingdom); anti-p21 monoclonal mouse IgG, CP74 (Sigma)] at 4°C overnight, washed with TBS-T, and incubated with a horseradish peroxidase-conjugated rabbit anti-goat IgG (for decorin) or goat antimouse IgG (for p21) for 1 h at room temperature. Signal detection was performed using an enhanced chemiluminescence reaction (ECL Western blotting detection; Amersham Life Science, Amersham, United Kingdom).

**Immunohistochemistry.** Paraffin-embedded pancreatic tissue sections (4-μm thick) obtained from patients with pancreatic cancer and normal donors were deparaffinized with xylene and rehydrated through graded alcohol into distilled water. After washing in Tris-buffered saline, endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide in methanol. To block unspecific activity of secondary antibodies, slides were treated with nonimmune normal rabbit serum (1:200 g/ml in normal rabbit serum), slides were washed with Tris-buffered saline and 0.05% Tween 20, treated with a 1:200 dilution of rabbit anti-goat horseradish peroxidase-labeled secondary antibodies (Sigma) for 1 h, developed using Dako Envision + System (DAKO Diagnostics AG, Zurich, Switzerland), and slightly counterstained with Mayer’s hematoxylin. To ensure antibody specificity, negative control slides were incubated either in the absence of primary antibody or with a normal goat IgG. All slides were analyzed by two independent observers blinded to patient status, followed by resolution of any differences by joint review and consultation with a third observer. In addition, double staining against smooth muscle actin (mouse anti-smooth muscle actin; Sigma) and decorin was performed.

**Statistical Analysis.** For statistical analysis, we used SPSS statistical software (version 11.0 for Windows). The Mann-Whitney test was used to estimate the significance of observed differences, and the Spearman rank test was used to establish correlation with histological and clinical parameters.

**RESULTS**

**Decorin Expression Is Highly Up-Regulated in Patients with Pancreatic Cancer.** Quantitative PCR was performed to evaluate the level of expression of decorin mRNA in normal pancreatic tissue samples and pancreatic cancer tissue samples. The tissue samples from normal controls had an average replicate number of decorin mRNA of 7079 ± 1000, whereas the average replicate number was increased 9-fold in patients with pancreatic cancer (63948 ± 8538; P = 0.0001). The distribution of real-time quantitative PCR data in patients with pancreatic cancer and control samples is summarized in Fig. 1A. Although decorin levels in the cancerous pancreas always exceeded those of normal donors, some patient-to-patient variability was observed. However, except for an association with abundant ECM present in all tumor specimens at the moment of resection, no correlation could be established between decorin expression and tumor stage, metastasis, or patient survival. Thus, decorin may represent a useful molecular marker of pancreatic cancer-associated desmoplastic reaction, but its prognostic value should be further evaluated in a bigger cohort of patients.

**Western blot analysis confirmed expression of decorin on a protein level in pancreatic cancer samples (Fig. 1B): a ladder of decorin-specific bands was detected in cancerous tissues, but not in normal ones.**

**Decorin Is Localized in the ECM but not in Tumor Cells.** To localize decorin protein in tissues, immunohistochemical analysis was performed in normal pancreas samples (Fig. 2A) and in pancreatic cancer samples (Fig. 2, B–D). In normal pancreatic tissue samples, weak decorin immunoreactivity was present in the ECM, and this was more evident around small ducts (Fig. 2A, d). Islet (Fig. 2A, inset, I) and acinar cells were devoid of any decorin signal.

In contrast, pancreatic cancer tissue sections exhibited intense decorin immunoreactivity. The staining signals were primarily localized in the ECM, especially at the borders of the tumor areas (Fig. 2, B and C). In contrast, pancreatic cancer cells exhibited no decorin immunoreactivity (Fig. 2C).
PSCs Represent the Source of Decorin in Pancreatic Cancer. Because myofibroblasts have been suggested to be the probable source of decorin in ovarian cancer (15), we hypothesized that pancreatic myofibroblasts (PSCs) might produce decorin as well. Therefore, double staining for decorin and smooth muscle actin was performed and showed PSCs embedded in areas of strong decorin immunoreactivity (Fig. 2D, inset). To confirm that PSCs are the origin of decorin in pancreatic cancer, primary cultures of PSCs were analyzed by reverse transcription-PCR for decorin mRNA expression. Decorin transcripts were detected in all tissue samples and PSCs (Fig. 3). To confirm the absence of decorin in pancreatic cancer cells, pancreatic cancer cell lines were analyzed for decorin RNA and protein expression by real-time quantitative PCR and Western blot analysis. Among the eight cancer cell lines analyzed, only AsPC-1 and T3M4 expressed decorin mRNA. The number of detectable copies was minuscule (<2/µl, compared with approximately 70,000 copies/µl in cancer), and no decorin protein could be detected (data not shown).

Responsiveness of Pancreatic Cancer Cells to the Antitumor Effect of Decorin. Next, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test, we investigated whether decorin exerts growth-stimulatory or growth-inhibitory effects on pancreatic cancer cell lines. After incubation with 100 µg/ml decorin, all pancreatic cancer cell lines showed responsiveness to decorin (Fig. 4). In all cancer cell lines, growth suppression could be observed, although sig-
significant reduction was only seen in T3M4, Colo-357, and BxPC-3. Of note, the effects of decorin on pancreatic cancer cell lines were only detectable if cells were grown on collagen-coated surfaces. As described previously for other types of cancer, incubation of pancreatic cancer cell lines with decorin led to p21-mediated G1 accumulation (Fig. 5, A and B).

Decorin Attenuates the Cytostatic Actions of Chemotherapeutic Drugs toward Pancreatic Cancer Cells. Carboplatin and gemcitabine were able to suppress pancreatic tumor cell growth in vitro (Fig. 6, A and B). Decorin attenuated the cytostatic effect of carboplatin on pancreatic cancer cells in five cell lines, up to a maximum of 67% in Colo-357. Synergism of decorin and carboplatin led to an additional growth suppression of 16% only in T3M4 cancer cells.

The cytostatic effect of gemcitabine, which is most commonly used in the treatment of pancreatic cancer, was also attenuated by decorin in five cancer cell lines, with a maximum attenuation of 50% in Colo-357. Again, synergism of decorin and gemcitabine led to an additional growth-suppressive effect of 19% only in T3M4.

DISCUSSION

The discovery that the fibril-associated proteoglycan decorin serves not only as an organizer of the ECM but is also a signaling molecule with antitumor properties led to a novel level of complexity in tumor cell-ECM interactions (35, 36). Decorin, a well-known member of the family of small leucinerich proteoglycans, was shown to control and suppress cancer growth and invasion, presumably by influencing the biological activity of growth factors such as TGF-β, platelet-derived growth factor, vascular endothelial growth factor, and epidermal growth factor, which are released from cancer cells (12, 14, 15, 26, 36).

Decorin expression is down-regulated in tumor cells of breast, colon, gastric, hepatocellular, and ovarian cancer but preserved in the surrounding ECM (5, 15, 24–28). Reduced decorin in the peritumoral stroma of breast cancer worsens the prognosis in node-negative patients (29). In contrast, up-regulation of decorin in tumor cells and/or extracellular tissue reduces cancer cell proliferation (8, 11, 17, 18, 30, 36). Hence, although transformed epithelial cells cease to produce decorin, they retain responsiveness to its effects. In the present analysis in pancreatic cancer, we found up-regulation of decorin on mRNA and protein levels in resected tumor specimens. However, statistical analysis did not reveal a positive or negative correlation between the net amount of decorin in tumor samples and tumor-node-metastasis (TNM) status, cancer grade, or patient survival.

In our study, pancreatic cancer cells themselves did not produce decorin, but they were responsive to the antiproliferative effects of this protein, albeit only on collagen-coated surfaces. Because decorin noncovalently binds to fibrillar collagen type I (37, 38), a protein that also has proliferation-inhibiting...
In pancreatic cancer, decorin attenuates the cytostatic activity of chemotherapeutic drugs. A, overnight cultures of pancreatic cancer cell lines grown on collagen-coated surfaces were exposed or not exposed to decorin (100 ng/ml), carboplatin (100 µg/ml), or a combination of both. Forty-eight h later, cell viability was assayed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. Data points are a percentage of the values obtained for untreated cells (control) and summarized the results obtained from three experiments. B, pancreatic cancer cell lines were also exposed to gemcitabine (12 ng/ml), or a combination of both. The decorin-mediated growth inhibition pathway over-organized the ECM, with possible trapping of chemotherapeutic agents (10, 12). A change in the microenvironment may also influence the behavior of malignant cells; therefore, decorin-mediated ECM alterations may alter the sensitivity of pancreatic cancer cells to chemotherapy (52).

In conclusion, our results show that decorin expression and production are up-regulated in pancreatic cancer during host reaction aimed at tumor sequestering and growth inhibition. The decorin-mediated growth inhibition pathway stays intact in malignant pancreatic cells, thus proposing this naturally occurring protein as a potential anticancer substance (once expressed in tumor cells or in close proximity to tumor cells). However, we show for the first time that the moderate antitumor effect of collagen-associated decorin would possibly be negated by its ability to attenuate the efficiency of chemotherapeutic agents. Whereas the level of decorin transcription may serve as a useful molecular marker of desmoplastic changes, the clinical prognostic value of decorin should be further investigated with regard to the impact of decorin on chemotherapy efficiency.

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