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Tumor Stroma Interactions Induce Chemoresistance in Pancreatic Ductal Carcinoma Cells Involving Increased Secretion and Paracrine Effects of Nitric Oxide and Interleukin-1β

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ABSTRACT

Pancreatic ductal carcinoma is characterized by a profound chemoresistance. As we have shown previously, these tumor cells can develop chemoresistance by interleukin (IL)-1β in an autocrine and nuclear factor-κB-dependent fashion. Because pancreatic ductal carcinoma contains many mesenchymal stromal cells, we further investigated how tumor–stroma interactions contribute to chemoresistance by using a transwell coculture model, including murine pancreatic fibroblasts and the chemosensitive human pancreatic carcinoma cell lines T3M4 and PT45-P1. If cultured with fibroblast-conditioned medium or kept in coculture with fibroblasts, both cell lines became much less sensitive toward treatment with etoposide than cells cultured under standard conditions. Furthermore, the secretion of IL-1β in T3M4 and PT45-P1 cells was increased by the fibroblasts, and IL-1β-receptor blockade abolished the resistance-inducing effect during cocultivation. This stimulated IL-1β secretion could be attributed to nitric oxide (NO) released by the fibroblasts as an IL-1β-inducing factor. Although both tumor cells secreted only little NO, which was in line with undetectable inducible nitric oxide synthase (iNOS) expression, fibroblasts exhibited significant iNOS expression and NO secretion that could be further induced by the tumor cells. Incubation of T3M4 and PT45-P1 cells with the NO donor S-Nitroso-*N*-acetyl-D,L-penicillamine up-regulated IL-1β secretion and conferred resistance toward etoposide-induced apoptosis. Conversely, the resistance-inducing effect of the fibroblasts was significantly abolished, when the specific iNOS inhibitor aminoguanidine was added during coculture. Immunohistochemistry on tissue sections from human pancreatic ductal carcinoma also revealed iNOS expression in stromal cells and IL-1β expression in tumor cells, thus supporting the *in vitro* findings. These data clearly demonstrate that fibroblasts contribute to the development of chemoresistance in pancreatic carcinoma cells via increased secretion of NO, which in turn leads to an elevated release of IL-1β by the tumor cells. These findings substantiate the implication of tumor–stromal interactions in the chemoresistance of pancreatic carcinoma.

INTRODUCTION

Pancreatic ductal carcinoma is characterized by a highly malignant phenotype associated with rapid tumor progression and metastasis (1, 2), and its treatment is highly limited by the innate or acquired resistance of pancreatic carcinoma cells toward chemotherapeutic drugs. One strategy by which tumor cells might acquire a chemoresistant phenotype is the protection from apoptosis. In this aspect, the transcription factor nuclear factor-κB (NF-κB) has gained much attention, because it is involved not only in tumor development and progression (3–5) but also in the induction of chemoresistance (6, 7). Elevated levels of NF-κB can lead to an up-regulation of antiapoptotic molecules, *e.g.*, members of the cellular inhibitor of apoptosis (cIAP) or Bcl-2 family (8, 9). Furthermore, an elevated NF-κB-binding activity can lead to the production and secretion of cytokines, which in turn serve as a stimulus for NF-κB activation. We have shown previously that pancreatic carcinoma cells can achieve a chemoresistant phenotype by secretion of interleukin (IL)-1β, thereby providing an autocrine mechanism of permanent activation of the transcription factor NF-κB (10).

Because it is known that many human cancers do not exist in isolation but rather intimately interact with non-neoplastic host cells (11), particular attention must be paid to the complex tumor–host interactions to fully understand how tumor cells become chemoresistant. Neoplasms like pancreatic carcinoma are typically composed of infiltrating adenocarcinoma surrounded by a predominant dense fibrous or desmoplastic stroma, which itself contains proliferating fibroblasts, small endothelial-lined vessels, inflammatory cells, and other components (12). Rather unique to ductal adenocarcinoma is a consistently low ratio of the infiltrating adenocarcinoma cells relative to the abundant desmoplastic stromal environment (13). Tumor cells can regulate the development of a "tumor stroma" by the aberrant expression of growth factors or induction of growth factor receptors in the stromal compartment. On its induction, the tumor stroma will reciprocally influence differentiation of the tumor cells. Therefore, continuous interactions between tumor cells and the stromal environment (resulting in their reciprocal regulation and modulation) are prerequisites for carcinoma development and progression by promoting migratory, invasive, and angiogenic abilities of the tumor cells (14).

Nitric oxide (NO) plays an important role in the regulation of tumorigenicity and metastasis (15, 16). It exists as a free radical, producing many reactive intermediates that account for its bioactivity, and it is generated by the conversion from L-arginine and molecular oxygen to L-citrulline by NO synthases (NOS). Three isoforms of the NOS exist: (*a*) the endothelial and (*b*) neuronal NOS are expressed constitutively and produce low amounts of NO that mediates physiological functions, such as neuronal transmission and vascular tone regulation (17, 18); and (*c*) in contrast, the inducible isoform (iNOS) is primarily expressed in activated macrophages, which often infiltrate tumors, but also by various types of cancer cells themselves (16, 19). High levels of NO, as secreted by activated macrophages, are described to have a more cytotoxic effect on tumor cells leading to the induction of apoptosis (20, 21), whereas low levels of iNOS and NO seem to be rather associated with tumor progression and metastasis (22). Therefore, the effects of NO on the apoptotic machinery seem to be complex and ambiguous, depending on its concentration. Still, little information exists: (*a*) whether and how the tumor microenvironment contributes to the acquisition of a chemoresistant phenotype in pancreatic carcinoma cells; and (*b*) which role NO plays in this scenario.

To study these questions, we set up an *in vitro* coculture model consisting of human pancreatic carcinoma cell lines and isolated

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murine pancreatic fibroblasts, thus distinguishing factors of fibroblastic (murine) and tumor (human) origin and their respective role in the phenomenon of chemoresistance. Our data clearly show that NO released by pancreatic fibroblasts lead to the up-regulation of IL-1β by pancreatic carcinoma cells and thereby to the induction of chemoresistance in these tumor cells. This work contributes essentially to the understanding of how chemoresistance might develop during tumor progression.

MATERIALS AND METHODS

Cell Lines and Culture. The human pancreatic carcinoma cell lines T3M4 and PT45-P1, as well as their handling, were described previously (23). All cell lines were kept in culture (37°C, 5% CO_2, and 85% humidity) using RPMI 1640 (PAA Laboratories, Cölbe, Germany), supplemented with 1% glutamine (Life Technologies, Inc., Eggenstein, Germany) and 10% FCS (Biochrom KG, Berlin, Germany).

Reagents and Antibodies. For blocking the action of human IL-1β, a human IL-1β receptor antagonist (IL-1βR-AG; R&D Systems, Wiesbaden-Nordenstadt, Germany) was used at a concentration of 125 ng/ml.

The NO donor S-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP) was purchased from Alexis (Grünberg, Germany) and used at a concentration of 20 μmmol/liter. The specific inhibitor of iNOS, aminoguanidine (AG), was obtained from Sigma-Aldrich Chemie (Schnelldorf, Germany) and used at a concentration of 2 mmol/liter. All reagents were added to mono or cocultured cells when medium was changed.

Isolation and Expansion of Murine Pancreatic Fibroblasts. Fibroblasts were isolated from the pancreas of female SCID mice formerly used in our *in vivo* experiments (24). After killing the mice, pancreata were removed and put in PBS (PAA Laboratories) with 2 μM trypsin inhibitor (Sigma-Aldrich Chemie). The organs were cut with a scalpel into pieces of 2 mm^3, and 25 of these pieces were put into a single Petri dish (10 cm; Sarstedt, Nümbrecht, Germany). To induce a migratory stimulus for the fibroblasts, each piece of tissue was put onto 2 μl of murine blood serum, which was mixed with 2 μl of 10 mM $CaCl_2$. After 15 min of coagulation, the tissue pieces were attached to the dish, and 12 ml of medium were added. For expansion, DMEM medium plus 1 gram/liter glucose was used (PAA Laboratories) containing 10 mmol/liter HEPES, 100 μg/ml gentamicin, 1% glutamine (all from Life Technologies), 100 μg/ml Penicillin/Streptomycin, and 20% FCS (both from Biochrom). Attached tissue pieces were cultured at 37°C, 5% CO_2, and 85% humidity. Medium was replaced every 2 days. After 15 days, when enough fibroblasts were migrated out of the tissue, cells were detached by Trypsin/EDTA (Life technologies) and additional scraping with a cell scraper (Sarstedt). After centrifugation, 5×10^5 cells were put into a 75-cm^2 culture flask (Sarstedt). For further cultivation, medium containing 10% FCS was used. Before fibroblasts were used for coculture experiments, an immunohistological staining for vimentin and α-smooth muscle actin was performed to verify the myofibroblastic phenotype of the cells.

Generation of Conditioned Media from Fibroblasts and Tumor Cells. To analyze the effects of secreted factors from fibroblasts on tumor cells and *vice versa,* cells were cultured with respective medium for 36–48 h. Before use, these conditioned media were precleared by centrifugation (10,000 rpm for 10 min) and used for additional experiments.

ELISA. Cells (1×10^5)/well were grown in a six-well culture plate. After 24 h, medium was replaced, and tumor cells were incubated either with standard medium or conditioned medium from fibroblasts. *Vice versa,* fibroblasts were incubated with standard medium or conditioned media from tumor cells. After 48 h, supernatants were taken for ELISA analysis.

In the transwell coculture system, 1×10^5 tumor cells were seeded into the bottom compartment of a six-well culture plate, and 2×10^5 fibroblasts were seeded into the top transwell compartment (Costar GmbH, Bodenheim, Germany). Medium was replaced after 24 h, and supernatants were taken after an additional 72 h of coculture for ELISA analysis. To determine levels of human IL-1β and NO secreted into cell culture supernatants, the Quantikine-HS human IL-1β immunoassay and Total NO colorimetric assay, respectively, were used (both from R&D Systems). The assays were performed following the instructions of the manufacturer. Before use, supernatants were precleared by centrifugation (10,000 rpm for 10 min). Concentrations of measured IL-1β and NO were normalized to the cell numbers determined in parallel. Presented data show the amount of NO over background levels measured in medium without cells.

Apoptosis Induction and Annexin V Staining. For induction of apoptosis, T3M4 and PT45-P1 cells were seeded into a six-well culture plate at a concentration of 1×10^5 cells/well. In some experiments, the medium was replaced either by standard medium or by conditioned medium of the fibroblasts after 24 h, and tumor cells were cultured for an additional 48 h. In the transwell coculture system, additionally to the tumor cells, 2×10^5 fibroblasts were seeded into the top transwell compartment. Medium was replaced after 24 h, and the coculture continued for an additional 72 h.

After 24 h of culture with conditioned medium, or after 48 h of coculture with fibroblasts, cells were either left untreated or treated with 20 μg/ml etoposide. After an additional 24 h, apoptosis was determined by staining with annexin V/propidium iodide (ApoAlert apoptosis assay; Clontech, Heidelberg, Germany) according to the instructions of the manufacturer. In coculture experiments, only tumor cells were analyzed for apoptosis. Analysis was done by fluorescence flow cytometry (GalaxyArgon Plus; DAKO Cytomation, Hamburg, Germany) using the FLOMAX software. Cells exhibiting high specific annexin V staining were regarded as apoptotic.

Western Blotting. Cells ($5–10 \times 10^6$)/well were seeded into a six-well culture plate. For cell preparation, medium was removed, and cells were washed once with PBS and then lysed with one volume of 2 × SDS sample buffer (128 mmol/liter Tris-Base, 4.6% SDS, and 10% glycerol). Samples were heated for 5 min at 95°C and put on ice for 2 min. Protein concentrations were determined by using the D_c Protein assay from Bio-Rad Laboratories (München, Germany). Equal amounts of protein (10 μg) were used of each sample, and an appropriate volume of SDS sample buffer containing 0.2 mg/ml bromphenolblue (Serva, Heidelberg, Germany) and 2.5% β-Mercaptoethanol (Biomol, Hamburg, Germany) was added. Samples were run on an 8% SDS-PAA gel, and immunoblotting was performed as described previously (6). For detection of iNOS, a monoclonal antibody (R&D Systems) was diluted at a concentration of 1 μg/ml in 2% nonfat milk powder and 0.05% Tween in Tris-buffered saline and incubated overnight at 4°C. As control of equal protein load, a monoclonal antibody for α-tubulin (Sigma-Aldrich Chemie) was diluted 1:5000 in 5% bovine serume albumine (Serva) and 0.05% Tween in Tris-buffered saline overnight at 4°C. For detection of the primary antibodies, an antimouse horseradish peroxidase-linked antibody (Cell Signaling via New England Biolabs GmbH, Frankfurt a.M., Germany) was used at a dilution of 1:2000 in 5% nonfat milk powder and 0.05% Tween in Tris-buffered saline at room temperature for 1 h.

Immunohistochemistry. Thin sections (5 μm) of 22 formalin-fixed, paraffin-embedded tissue blocks from human pancreatic ductal adenocarcinoma were deparaffinized and rehydrated. Heat-mediated antigen retrieval was carried out in pressure cooker in PBS for 5 min followed by blocking with 3% H_2O_2 in methanol solution. The primary mouse monoclonal anti-iNOS antibody (C-11; Santa Cruz Biotechnology, Heidelberg, Germany) was applied at 13 μg/ml concentration overnight at 4°C. Mouse Envision Kit was used for detection according to the manufacturer's instructions (DAKO Cytomation). For IL-1β immunostaining, 20 pancreatic ductal carcinoma samples obtained from surgical specimens according to a protocol approved by the ethics committee of the University Hospitals, Kiel (permission number 110/99) were used. Samples were snap frozen in a mixture of isopentane and dry ice and stored at −80°C. Thin cryostat sections (5 μm) were cut, air dried, and fixed in acetone at room temperature for 10 min and than incubated with 0.03% hydrogen peroxide (DAKO Cytomation) four times for 20 min. Intrinsic avidin and biotin were blocked with the Avidin-Biotin Blocking Kit (Vector Laboratories via Alexis). Serum blocking and detection were performed with a peroxidase-based rabbit Vectastain Kit (Vector Laboratories). Rabbit polyclonal anti-IL-1β antibody (C-20, Santa Cruz Biotechnology) was applied in 0.35 μg/ml concentration 1 h at room temperature. For negative control, both iNOS and IL-1β antibodies were coincubated with 5-fold weight excess of specific blocking peptides for 1 h before immunostaining. After staining, sections were counterstained in 50% hemalaun (Merck) and mounted with glycerol gelatin. Immunohistochemical stainings were evaluated by a semiquantitative method. iNOS and IL-1β expression in tumor and stromal cells were rated as mild (<10% of cells stained), moderate (10–50%), or strong (>50%).

RESULTS

Fibroblasts Induce Chemoresistance in T3M4 and PT45-P1 Cells. We have shown previously that pancreatic carcinoma cells are able to maintain a chemoresistant phenotype by autocrine secretion of IL-1β (10). Because pancreatic carcinoma largely consists of stromal fibroblasts (12, 13), the role of these cells in the development of chemoresistance was investigated. As representatives of chemosensitive human pancreatic carcinoma cell lines, we used T3M4 and PT45-P1 cells that were cultured with conditioned medium from expanded murine pancreatic fibroblasts or under standard conditions with medium. After 24 h, cells were treated with 20 μg/ml etoposide for an additional 24 h, and the number of apoptotic tumor cells was determined by annexin V staining and fluorescence-activated cell sorter analysis. As shown in Fig. 1*A*, when cultured in standard medium, apoptosis could be induced in 25% of T3M4 cells and 41% of PT45-P1 cells, indicating that both cell lines were sensitive toward treatment with etoposide. In contrast, conditioned medium of fibroblasts rendered these cells resistant toward cytostatic drug treatment so that only 4% of T3M4 and 17% of PT45-P1 cells were still sensitive to etoposide. When tumor cells were incubated with fibroblasts in the transwell coculture system for 72 h, sensitivity toward etoposide was also decreased in comparison with monocultured cells, in T3M4 cells from 28 to 14.5%, and in PT45-P1 from 63 to 31% (Fig. 1*B*). Similar results were obtained by using the M30-apoptosense assay, which specifically detects a neo-epitope of cytokeratine 18 and which is only exposed after cleavage of caspases in cells of epithelial origin (data not shown). These results demonstrate that pancreatic fibroblasts secrete factors which lead to chemoresistance in pancreatic carcinoma cells.

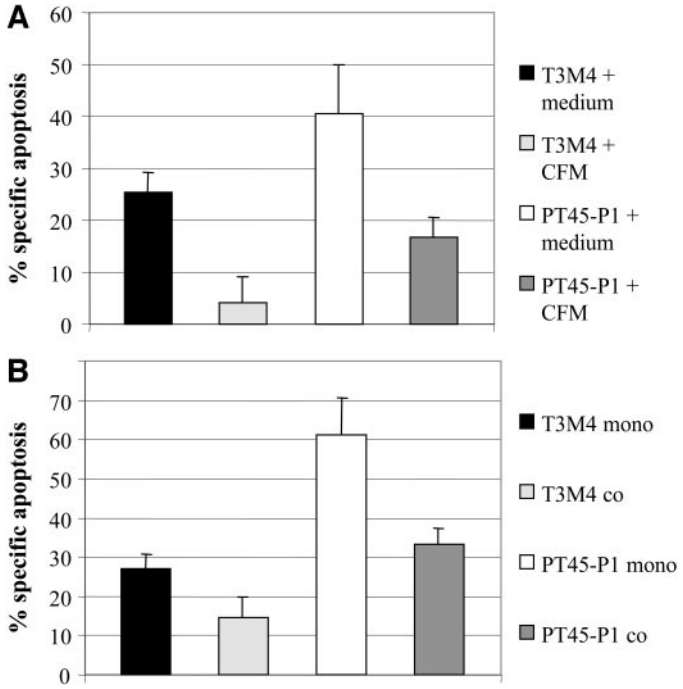

Fig. 1. Fibroblasts induce chemoresistance in T3M4 and PT45-P1 cells. In *A*, T3M4 and PT45-P1 cells were seeded into six-well plates, and after 24 h, medium was replaced. Cells were then either cultured with standard medium or conditioned medium of fibroblasts (*CFM*) for 48 h. After 24 h, cells were either left untreated or treated with 20 μg/ml etoposide. In *B*, T3M4 and PT45-P1 cells were either monocultured (*mono*) or cocultured with fibroblasts (*co*). After 48 h, cells were either left untreated or treated with 20 μg/ml etoposide. After an additional 24 h, tumor cells were stained with annexin V and analyzed by fluorescence flow cytometry. The basal level of apoptosis was 15–25% for T3M4 cells and 5–10% for PT45-P1 cells. Data are presented as percentage-specific apoptosis over basal level. Means and SDs from three independent experiments are shown.

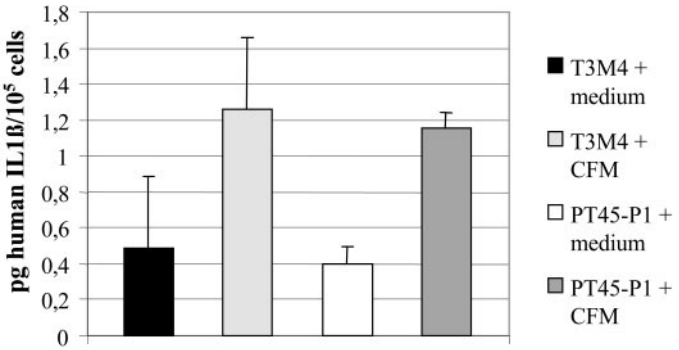

Fig. 2. Fibroblasts induce secretion of interleukin (*IL*)-1β by T3M4 and PT45-P1 cells. T3M4 and PT45-P1 cells were seeded into six-well plates, and after 24 h, medium was replaced. Cells were then either cultured with standard medium or with conditioned medium of fibroblasts (*CFM*) for 48 h. Supernatants were then cleared and subjected to a commercial IL-1β immunoassay. The amount of IL-1β was normalized to equal cell number, which was determined in parallel (expressed as pg human IL-1β/10^5 cells). Data represent the means and SDs from four independent experiments.

Fibroblasts Induce Secretion of IL-1β by T3M4 and PT45-P1 Cells. Because IL-1β was identified as a chemoresistance-inducing factor (10) in pancreatic carcinoma cells, we investigated whether chemoresistance induced by fibroblasts involves increased secretion of IL-1β by the tumor cells. As verified by an immunoassay for murine IL-1β, fibroblasts did not secrete any IL-1β, and its secretion was neither elevated by coculture with T3M4 and PT45-P1 cells, respectively, nor by culture with conditioned medium of both tumor cell lines (data not shown). As shown in Fig. 2, T3M4 and PT45-P1 cells, cultured in normal medium, secreted only low levels of IL-1β (0.4 pg/10^5 cells), but secretion was induced 3-fold by culture with conditioned medium of fibroblasts. Because the immunoassay detects only human IL-1β and is not cross-reactive to murine IL-1β, it can be excluded that the determined levels of IL-1β were secreted by the murine fibroblasts. These data show that an interaction with fibroblasts leads to an elevated secretion of IL-1β by T3M4 and PT45-P1 cells.

Fibroblast-Induced IL-1β Secretion by T3M4 and PT45-P1 Cells Accounts for Chemoresistance. Next, we investigated whether the elevated IL-1β secretion is responsible for fibroblast-induced resistance toward cytostatic drugs; T3M4 and PT45-P1 cells were cultured either with normal medium or with conditioned medium of fibroblasts, in the presence or absence of a human IL-1βR antagonist for 48 h. After 24 h, apoptosis was induced by 20 μg/ml etoposide, and the number of apoptotic cells was determined after 24 h of cytostatic drug treatment. Incubation of T3M4 and PT45-P1 cells with the IL-1βR antagonist hardly influenced the number of apoptotic cells when cells were cultured with medium (Fig. 3), as expected from the low basal IL-1β levels. In contrast, after culture with conditioned medium of fibroblasts, the presence of the IL-1βR antagonist significantly increased the rate of apoptosis in T3M4 cells from 4 to 21% and in PT45-P1 cells from 16 to 31%. These data clearly demonstrate that the chemoresistance-inducing effect of the fibroblasts depends on induced autocrine IL-1β secretion by T3M4 and PT45-P1 cells.

iNOS Expression and NO Secretion by Fibroblasts but not by Tumor Cells. It has been demonstrated that under certain conditions, pancreatic carcinoma shows significant iNOS expression and increased levels of NO (25). We therefore investigated the role of this signaling mediator in chemoresistance and its possible connection to the elevated secretion of IL-1β by the tumor cells. The inducible form of NOS was not expressed by pancreatic carcinoma cell lines T3M4 and PT45-P1 nor was it inducible by stimulation with 10 ng/ml IL-1β or by conditioned medium of the fibroblasts (data not shown). In contrast, fibroblasts showed a low basal expression of iNOS that was

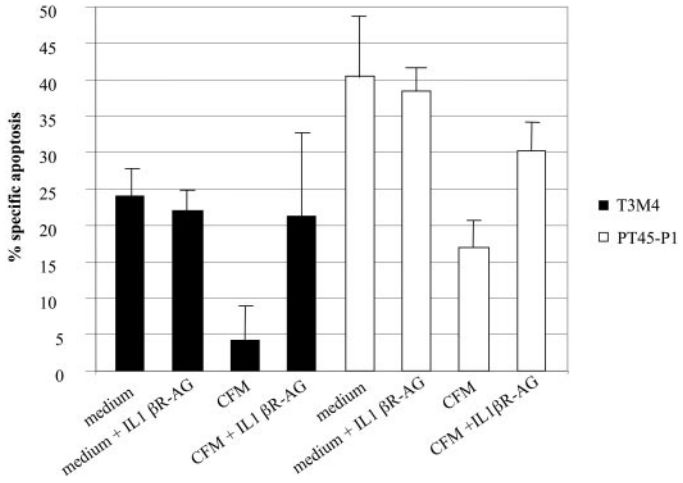

Fig. 3. Fibroblast-induced interleukin (*IL*)-1β secretion by T3M4 and PT45-P1 cells accounts for chemoresistance. T3M4 and PT45-P1 cells were seeded into six-well plates, and after 24 h, medium was replaced. Cells were then either cultured with standard medium or conditioned medium of fibroblasts (*CFM*) for 48 h, and to some samples, 125 ng/ml of the IL-1βR antagonist (*IL-1βR-AG*) were added for the time of culture. After an additional 24 h, cells were either left untreated or treated with 20 μg/ml etoposide. After 24 h, cells were stained with annexin V and analyzed by fluorescence flow cytometry. The basal level of apoptosis was 15–25% for T3M4 cells and 5–10% for PT45-P1 cells. Control experiments showed that the IL-1βR antagonist itself had no toxic effect on the tumor cells. Data are presented as percentage-specific apoptosis over basal level. Means and SDs from three independent experiments are shown.

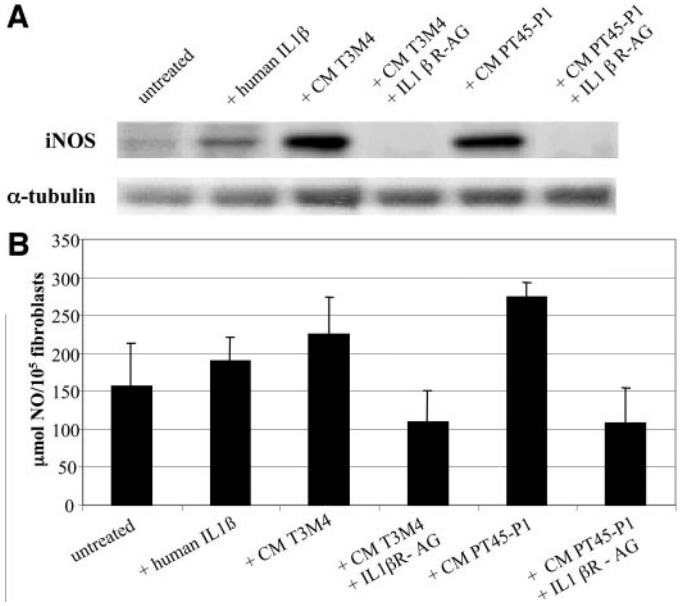

Fig. 4. Inducible nitric oxide synthase (*iNOS*) expression and nitric oxide (*NO*) secretion by fibroblasts. In *A*, Western blotting for the detection of iNOS was performed with whole cell lysates of fibroblasts, which were left untreated or treated with 10 ng/ml human interleukin (*IL*)-1β or conditioned medium (*CM*) of tumor cells for 48 h. Fibroblasts were also cultured with conditioned medium of tumor cells (*CM*) and an IL-1βR antagonist. α-tubulin was used as a control for equal protein load. In *B*, for detection of NO secreted by fibroblasts, cells were seeded into six-well plates, and after 24 h, medium was replaced. Fibroblasts were then either cultured with standard medium or conditioned medium (*CM*) of tumor cells for 48 h, in the absence or presence of an IL-1βR antagonist. As a control for NO induction, cells were treated with 10 ng/ml human IL-1β. Supernatants were then cleared and subjected to a commercial NO immunoassay. The amount of NO was normalized to equal cell number, which was determined in parallel (expressed as NO/10^5 μmol fibroblasts). Data represent the means and SDs from three independent experiments.

strongly up-regulated by IL-1β, as well as by conditioned medium of T3M4 and PT45-P1 cells (Fig. 4*A*). The detection of NO by ELISA revealed that T3M4 and PT45-P1 cell lines secreted only low amounts of NO, and this release could only marginally be increased by IL-1β or supernatants of fibroblasts (data not shown). In contrast, fibroblasts secreted high amounts of NO (152 μmol/10^5 cells), and this secretion was further up-regulated either by IL-1β or on incubation with conditioned medium of T3M4 and PT45-P1 cells (Fig. 4*B*). Usage of an IL-1βR antagonist during incubation with the tumor-conditioned media abolished up-regulation of iNOS expression (Fig. 4*A*), as well as NO release by the fibroblasts (Fig. 4*B*). These results show that fibroblasts produce high levels of NO, which is further elevated by the presence of pancreatic carcinoma cells and that tumor-derived IL-1β is one factor responsible for enhanced iNOS expression and NO release.

The NO Donor SNAP Induces Chemoresistance and IL-1β Secretion in T3M4 and PT45-P1 Cells. To investigate whether NO is able to induce chemoresistance and it is involved in the induction of IL-1β by the tumor cells, T3M4 and PT45-P1 cells were treated with the NO donor SNAP, and its impact on etoposide-induced apoptosis was measured. Treatment with SNAP led to NO levels corresponding to those achieved by culture with conditioned medium of fibroblasts or in the transwell coculture system (data not shown). Furthermore, treatment with SNAP increased IL-1β secretion in T3M4 cells >3-fold and in PT45-P1 cells by 50% (Fig. 5*A*). In comparison, conditioned medium of fibroblasts led to a 3-fold enhanced IL-1β secretion in both pancreatic carcinoma cells. Fig. 5*B* shows that SNAP was able to reduce the number of apoptotic T3M4 cells from 25 to 12%. This effect was even more pronounced in PT45-P1 cells, where SNAP reduced apoptosis from 41 to 17%, mimicking the resistance-inducing effect of the fibroblasts. In comparison, 4% of T3M4 and 16% of PT45-P1 cells were apoptotic when cultured with conditioned medium of fibroblasts. In all experiments, SNAP alone had no toxic effects on both tumor cell lines (data not shown). These findings demonstrate that NO is able to induce IL-1β in T3M4 and PT45-P1 cells, which thereby become resistant toward etoposide treatment.

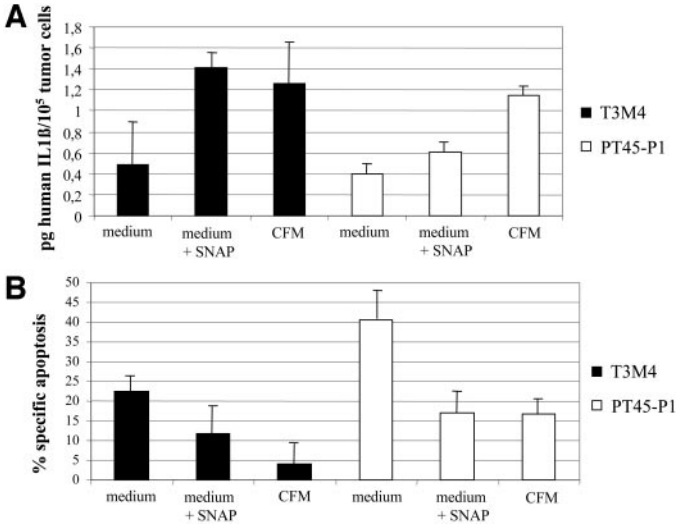

Fig. 5. The nitric oxide donor S-Nitroso-*N*-acetyl-D,L-penicillamine (*SNAP*) induces chemoresistance and interleukin (*IL*)-1β secretion in T3M4 and PT45-P1 cells. In *A*, for detection of IL-1β, T3M4 and PT45-P1 cells were seeded into six-well plates, and after 24 h, medium was replaced. Cells were then either cultured with standard or conditioned medium of fibroblasts for 48 h. To some cells which were cultivated with standard medium, 20 μmol/liter SNAP were added for time of culture. After 24 h, cells were either left untreated or treated with 20 μg/ml etoposide. After an additional 24 h, supernatants from T3M4 and PT45-P1 cells were collected, cleared, and subjected to a commercial IL-1β immunoassay. The amount of IL-1β was normalized to equal cell number, which was determined in parallel (expressed as pg human IL-1β/10^5 tumor cells). Data represent the means and SDs from three independent experiments. In *B*, for determination of apoptosis, cells were cultured and treated as described above. After 24 h of cytostatic drug treatment, cells were stained with annexin V and analyzed by fluorescence flow cytometry. The basal level of apoptosis was 15–25% for T3M4 cells and 5–10% for PT45-P1 cells. Data are presented as percentage-specific apoptosis over basal level. Means and SDs from three independent experiments are shown.

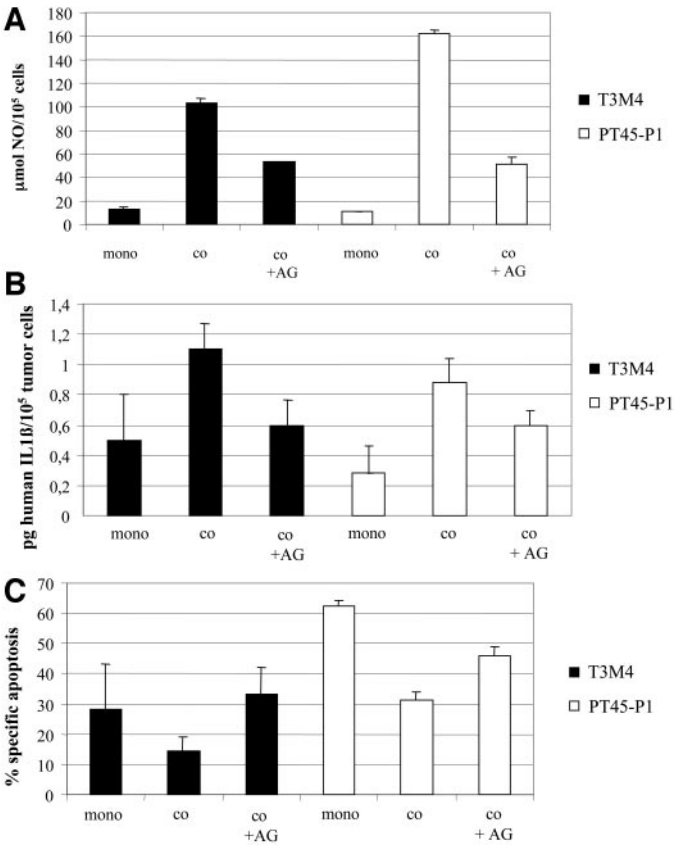

Fig. 6. Inhibition of inducible nitric oxide (*NO*) synthase reduces interleukin (*IL*)-1β secretion by tumor cells and abolishes the chemoresistance-inducing effect of fibroblasts. T3M4 and PT45-P1 were either cultivated alone (*mono*) or cultured in the presence of fibroblasts in a coculture system (*co*). After 24 h, medium was replaced, and 2 mmol/liter aminoguanidine (*AG*) were added to the samples as indicated. Culture lasted for 72 h. For detection of NO (*A*) and IL-1β (*B*), supernatants from mono and cocultures were collected, cleared, and subjected to a commercial NO and IL-1β immunoassay, respectively. The amount of NO was normalized to equal number of the total cell count (fibroblasts and tumor cells) and expressed as NO/10^5 μmol cells, and IL-1β was normalized to equal tumor cell number and expressed as pg human IL-1β/10^5 tumor cells. Data represent the means and SDs from three independent experiments. In *C*, for determination of apoptosis, tumor cells were stained with annexin V and analyzed by fluorescence flow cytometry after treatment with 20 μg/ml etoposide for 24 h. The basal level of apoptosis was 15–25% for T3M4 cells and 5–10% for PT45-P1 cells. Data are presented as percentage-specific apoptosis over basal level. Means and SDs from three independent experiments are shown.

Inhibition of iNOS Reduces IL-1β Secretion in Tumor Cells and Abolishes the Chemoresistance-Inducing Effect of Fibroblasts. To support the hypothesis that NO is an IL-1β-inducing factor which contributes to chemoresistance of pancreatic carcinoma cells, NO release was blocked by inhibition of iNOS activity. T3M4 and PT45-P1 cells were either cultured alone (monoculture) or cocultured with fibroblasts for 72 h. After 48 h, when cells were either left untreated or treated with the specific iNOS inhibitor AG, 20 μg/ml etoposide were added for 24 h. Fig. 6*A* shows that the elevated NO levels in the coculture of fibroblasts with T3M4 (103 μmol/10^5 cells) and PT45-P1 cells (160 μmol/10^5 cells) were significantly reduced to 53 μmol/10^5 cells in the coculture with T3M4 and to 52 μmol/10^5 cells in the coculture with PT45-P1 cells by treatment with AG. In parallel, levels of IL-1β were also reduced by the iNOS inhibitor (Fig. 6*B*). In cocultured T3M4 cells, treatment with AG reduced the IL-1β levels from 1.1 to 0.6 pg/10^5 cells, and in cocultured PT45-P1, the amount of released IL-1β was decreased from 0.9 to 0.6 pg/10^5 cells. Consistent with these data, the number of apoptotic cocultured T3M4 and PT45-P1 cells could be increased by treatment with AG (Fig. 6*C*). While apoptosis could only be induced in 14% of T3M4 cells and 31% of PT45-P1 cells when cocultured with fibroblasts, the number of apoptotic cells increased significantly to 33% (T3M4 cells) and 46% (PT45-P1 cells) after additional treatment with AG. This was not caused by a cytotoxic effect of the iNOS inhibitor, because this substance rather reduced the number of apoptotic tumor cells when these were cultured in the absence of fibroblasts (monoculture). AG itself had also no toxic effects on the cells, neither in mono nor in coculture. Furthermore, it had no significant effects on NO and IL-1β secretion by monocultured T3M4 and PT45-P1 cells, reflecting the low basal levels of both signaling mediators in these tumor cells (data not shown).

In summary, these results clearly demonstrate that NO is able to induce IL-1β and thereby resistance toward etoposide in T3M4 and PT45-P1 cells.

iNOS and IL-1β Expression in Human Pancreatic Ductal Adenocarcinoma. To further support the relevance of our *in vitro* findings, sections of human pancreatic ductal carcinomas were stained for iNOS and IL-1β. In 16 of 22 tumor specimens, iNOS expression was detected in fibroblasts (mild in 12 and moderate in 4 cases) that were frequently located in the proximity of tumor cells and in the tumor cells themselves (Fig. 7*A*). To a lesser extent, specific iNOS expression was also detected in endothelial cells and inflammatory cells, such as macrophages and neutrophil granulocytes. In 12 of 20 tumor specimens, IL-1β was detected in neoplastic cells (mild in 3, moderate in 6, and strong in 3 cases; Fig. 7*C*). Fibroblasts of the tumor stroma (Fig. 7*C*) and peritumoral fibrosis exhibited also IL-1β expression, as well as some endothelial cells and peripheral nerves. These findings demonstrate that the observed autocrine and paracrine mechanisms of chemoresistance *in vitro* might also be present in patients with pancreatic ductal adenocarcinoma.

DISCUSSION

Chemoresistance is still the major problem of anticancer drug treatment of malignant diseases, such as pancreatic carcinoma. To elucidate the contribution of tumor–stroma interactions to resistance against anticancer drug-induced apoptosis, we used a coculture model of human pancreatic carcinoma cells and murine pancreatic fibroblasts. As we could demonstrate for the first time, this interaction provides a mutual amplification loop contributing to chemoresistance of pancreatic carcinoma cells and involving IL-1β-triggered NO secretion by the fibroblasts and NO-dependent IL-1β secretion by the tumor cells.

Fibroblasts were freshly isolated from murine pancreas and expanded as a representative model for stromal cells, which can be associated with an adenocarcinoma. These cells exhibited the characteristic phenotype of myofibroblasts (26), such as expression of vimentin and α-smooth muscle actin, large size, numerous cytoplasmic extensions, and slow growth (data not shown). Because it is known that ductal adenocarcinoma contains a consistently low ratio of the adenocarcinoma component compared with the abundant desmoplastic stromal environment (17), we used a tumor–stroma ratio of 1:2 in our model system.

Culture of two sensitive human pancreatic carcinoma cell lines, T3M4 and PT45-P1, together with fibroblasts in a transwell coculture system or in conditioned medium of these fibroblasts led to a significant protection from etoposide-induced apoptosis (Fig. 1). This resistance-inducing effect was not caused by serum consumption by the fibroblasts, and the growth rate of cocultured T3M4 or PT45-P1 cells was only marginally decreased (data not shown), thus not accounting for decreased chemosensitivity. Interestingly, other tumor cell lines, such as 293, HeLa, or Colo320, did not gain increased resistance if cocultured with fibroblasts and were also

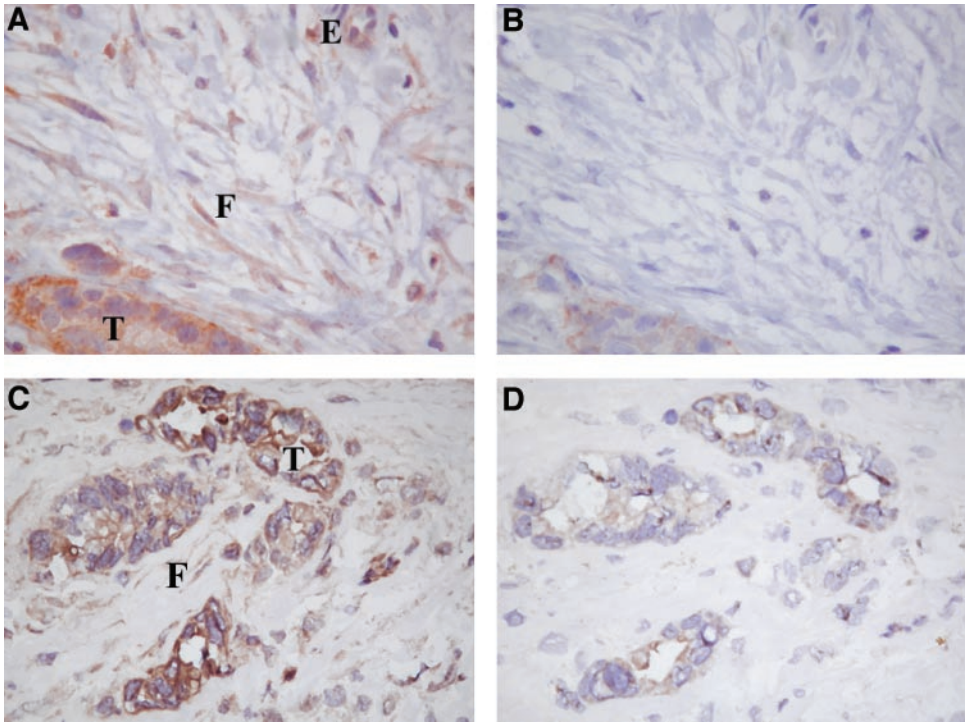

Fig. 7. Immunohistochemical detection of inducible nitric oxide synthase and interleukin-1β in human pancreatic ductal adenocarcinoma. Immunohistochemical staining for inducible nitric oxide synthase and interleukin-1β in human pancreatic ductal adenocarcinoma. In *a*, strong inducible nitric oxide synthase expression was detected in fibroblasts (*F*), tumor cells (*T*), and endothelial cells (*E*), as well. In *b*, preabsorption of the inducible nitric oxide synthase antibody with a specific blocking peptide before stain eliminated the signal almost completely. In *c*, intensive interleukin-1β expression was found in tumor cells (*T*) but also in fibroblasts (*F*) in the vicinity of tumor cells. In *d*, preabsorption of the interleukin-1β antibody with a specific blocking peptide before stain eliminated the signal almost completely.

not capable of inducing elevated NO secretion by fibroblasts (data not shown).

Thus, more specific factors secreted by the pancreatic tumor cells on the one hand, as well as by the fibroblasts on the other hand, might be responsible for the resistance-inducing effect of the coculture. As we could demonstrate, the resistance-inducing effect of the fibroblasts involves an increased IL-1β secretion by pancreatic tumor cells. Although these two chemosensitive cell lines normally secrete only low levels of IL-1β (Fig. 2), they gain chemoresistance by an IL-1β-dependent mechanism, as it has been shown recently for several resistant pancreatic carcinoma cell lines (10). Interestingly, the low basal IL-1β secretion by T3M4 and PT45-P1 cells does not confer chemoresistance, but it is obviously sufficient for an induction of fibroblast-derived NO, because it is a strong stimulus for the induction of iNOS (Fig. 4), whose enzymatic activity leads to NO production (21). In contrast to other reports, we detected neither iNOS nor significant amounts of secreted NO in both pancreatic carcinoma cell lines, neither constitutively nor on treatment with conditioned medium from fibroblasts (data not shown). In contrast, fibroblasts showed significant iNOS expression and secretion of NO, which was further enhanced by human IL-1β and conditioned medium of T3M4 and PT45-P1 cells (Fig. 4). In turn, NO, either secreted by fibroblasts or derived from the NO donor SNAP, induced a significant up-regulation of IL-1β secretion in T3M4 and PT45-P1 cells, hereby leading to chemoresistance of both tumor cell lines (Fig. 5). Blocking of NO release by inhibition of iNOS with the specific inhibitor AG decreased IL-1β secretion by the tumor cells and abolished the resistance-inducing effect of the fibroblasts (Fig. 6).

These findings could be confirmed by immunohistochemical stainings of tissue sections from pancreatic adenocarcinomas. iNOS expression was frequently (>70%) found in tumor-surrounding fibroblasts but also in tumor, endothelial, and inflammatory cells (Fig. 7*A*). IL-1β expression was detected in tumor cells in 60% of the carcinomas (Fig. 7*C*), and in addition, IL-1β expression was also found in fibroblasts (Fig. 7*C*), endothelial cells, and peripheral nerves. These findings are somewhat different from the coculture situation but might reflect a further progression of the described paracrine amplification loop, which involves not only tumor cells and fibroblasts but also other cells being present in the stromal compartment (*e.g.*, endothelial and inflammatory cells).

According to our data, NO contributes importantly to the development of chemoresistance in pancreatic carcinoma by inducing IL-1β secretion in the tumor cells that provides protection from anticancer drugs. This paracrine-positive feedback loop involves the activation of NF-κB, as demonstrated recently for the autocrine action of IL-1β (10), but other transcription factors might also be involved in the activation of NO and IL-1β, *e.g.*, AP-1 (27) or IRF-1 (28). The effects of NO on the apoptotic machinery are complex, *e.g.*, depending on its concentration. High levels of NO, as secreted by activated macrophages, have been described to exert cytotoxic effects on tumor cells leading to the induction of apoptosis (20, 21). In contrast, Zhao *et al.* (29) showed that moderate iNOS activity and NO release induced by CD23 stimulation inhibits apoptosis in B-cell chronic lymphocytic leukemia cells, which might be attributable to inhibition of caspase activity (30), as it has recently been shown by Zeigler *et al.* (31). This group demonstrates that NO blocks the activity of caspase-3 and -9 by nitrosylation of thiol residues, thereby promoting survival of human monocytes. Yang *et al.* (32) also report an antiapoptotic effect of NO in glioma cells on cytostatic drug treatment with 1,3-bis(2-chloro-ethyl)-1-nitrisourea and 1-(2-chloroethyl)–3-cyclohexyl-1-nitrosourea but not by cisplatin. In comparison, NO apparently protects pancreatic carcinoma cells from apoptosis induced by different cytostatic drugs. Although etoposide, gemcitabine, and cisplatin (data not shown) exert their cytotoxic effects by different modes of action, NO seems to inhibit apoptosis via IL-1β at a common site in the signaling cascade of these tumor cells. The exact unique target point of IL-1β in the apoptotic machinery is under current investigation, but it seems that "classical" antiapoptotic genes, such as *bcl-2* and *bcl-xl*, are not substantially up-regulated by the IL-1β-induced NFκB activation in our experimen-

tal setting.[3] In summary, these data contribute essentially to the understanding in chemoresistance of pancreatic carcinoma cells and underscore the importance of the tumor environment in the process of tumorgenecity, progression, and cancer treatment.

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