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Chronic Pancreatitis and Systemic Inflammatory Response Syndrome Prevent Impact of Chemotherapy with Gemcitabine in a Genetically Engineered Mouse Model of Pancreatic Cancer[1,2]

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Abstract

BACKGROUND AND AIMS: Gemcitabine is the standard therapy for patients with pancreatic cancer with metastatic disease. Patients with metastatic pancreatic cancer presenting with increased values of C-reactive protein do not respond to gemcitabine. So far, no studies have evaluated the correlation between chronic pancreatitis, systemic inflammatory response syndrome, and the loss of chemotherapeutic benefit. *METHODS:* Pdx-1-Cre;LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$ mice were assigned into four groups: 1) Sixteen animals received a daily intraperitoneal injection of caerulein from their ninth week of life on. 2) Sixteen mice were additionally given gemcitabine. 3) Twelve animals received gemcitabine only. 4) Saline-treated control group. Furthermore, human Paca44 pancreatic ductal adenocarcinoma cells were seeded and cultured in 0.5% FBS containing growth medium plus/minus 1 μM gemcitabine plus/minus recombinant human interleukin (IL)-6. *RESULTS:* Induced systemic inflammatory response syndrome and a mild chronic pancreatitis diminished the beneficial effects of gemcitabine upon median overall survival. In median, the monogemcitabine group survived 191 days, whereas the caerulein-mono group survived 114, the control group 121, and the caerulein gemcitabine group 127 days ($P < .05$). *In vitro*, the induction of STAT3 phosphorylation by recombinant human IL-6 promoted pancreatic ductal adenocarcinoma cell survival during gemcitabine treatment. *CONCLUSION:* We could demonstrate for the first time that an improvement in median overall survival with gemcitabine is significantly abolished by a persistent mild chronic pancreatitis and a systemic inflammatory response syndrome. In particular, the inflammation biomarkers C-reactive protein, IL-6, and IL-1α could indicate the prognostic benefit of gemcitabine chemotherapy and should now be tested in prospective patient-controlled trials.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths [1]. Unfortunately, the majority of patients presenting with the disease are already in an unresectable state at the time of diagnosis due to locally advanced or metastatic spread. Gemcitabine became the reference regimen for advanced pancreatic cancer after a randomized trial showed significant improvement in the median overall survival as compared with fluorouracil administered as an intravenous bolus (5.6 vs 4.4 months, $P = .002$) [2]. Numerous phase 3 trials of gemcitabine in combination with different cytotoxic or molecularly targeted agents have resulted in no substantial clinical improvement over the use of gemcitabine alone [3–8]. Only the addition of erlotinib

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to gemcitabine resulted in a significant but very small improvement in overall survival [9]. Last year, Conroy et al., demonstrated that a combination chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) as compared with gemcitabine as first-line therapy in patients with metastatic pancreatic cancer was associated with a survival advantage but had increased toxicity [10]. Still, the prognosis remains lethal. The efficacy of chemotherapy is still poor, and in some patients, systemic condition rapidly deteriorates after chemotherapeutic failure.

A systemic inflammatory response syndrome, marked by elevated circulating concentrations of multiple cytokines such as interleukin-6 (IL-6) or C-reactive protein (CRP), has been shown to be a disease-independent prognostic factor in a variety of tumors [11,12]. CRP is produced by the liver and is induced by proinflammatory cytokines, such as IL-6 or tumor necrosis factor-α [13], which are involved in cachexia. An elevated CRP concentration has previously been shown to have independent prognostic value in patients with PDAC [14,15]. So far, the mechanisms behind these observed phenomena are neither known nor analyzed.

Signal transducer and activator of transcription (STAT) proteins are present in the cytoplasm under basal conditions and are activated by phosphorylation on a single tyrosine residue. Activation of STAT3 depends on the phosphorylation of a tyrosine residue by upstream kinases, such as Janus kinase 2. Janus kinase 2 activation itself requires activation of the ubiquitin pathway by specific ligands, e.g., different interleukins. Of these ligands, IL-6 is one of the strongest activators of STAT3 [16]. STAT3 has been identified as a key oncogenic factor in a number of epithelial malignancies and is required for oncogenesis in mouse models of skin and gastric cancers [17,18]. In the pancreas, STAT3 is dispensable for normal development, whereas the majority of PDAC show constitutive activation of STAT3 [19].

The question remains what is the reason for patients being refractory to gemcitabine chemotherapy who present elevated CRP levels reflecting a systemic inflammatory immune response syndrome. In this study, we used a genetically engineered mouse model of PDAC which recapitulates human invasive pancreatic cancer on a genetic and histomorphologic level to address this question. We now show for the first time that gemcitabine will not have any effect on survival if a mild chronic pancreatitis and a consecutive systemic inflammatory response are induced in mice during chemotherapy. Furthermore, we found that IL-6 activates p-STAT3 which leads to increased chemoresistance in PDAC cells.

Material and Methods

Mice

Conditional *LSL-Trp53^{R172H}* [20], *LSL-Kras^{G12D}*, and *Pdx1-Cre* [21] strains were interbred to obtain *LSL-Kras^{G12D};LSL-Trp53^{R172H};Pdx1-Cre* triple-mutant animals on a mixed 129/SvJae/C57Bl/6 background as previously described [22]. All mice were generated from the same initial stock. All experiments were approved by the local committees for animal care and use. Animals were maintained in a climate-controlled room kept at 22°C, exposed to a 12:12-hour light–dark cycle, fed standard laboratory chow, and given water *ad libitum*.

Genotyping

For genotyping, genomic DNA was extracted from tail cuttings using the REDExtract-N-Amp Tissue polymerase chain reaction (PCR) kit (Sigma-Aldrich, St. Louis, MO). Three PCRs were carried out for each animal to test for the presence of the oncogenic Kras (using LoxP primers), p53, and Pdx1-Cre transgene constructs (using Cre-specific primers along with Gabra as positive control), respectively.

Drug Treatment

Transgenic Pdx-1-Cre;LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$ mice were randomly assigned into four groups: 1) To induce a chronic pancreatitis and a consecutive systemic inflammatory response syndrome, 16 animals received a daily intraperitoneal injection of 5 μg caerulein from their ninth week of life until death. 2) Sixteen mice were additionally given 2.5 mg gemcitabine weekly from their 13th week of life until death. 3) Twelve animals received only gemcitabine from their 13th week of life until death. 4) In addition, a saline-treated group was performed as control (n = 16) (Figure 1). In cases where littermates were available for drug treatment, only the first mouse was randomly assigned to one of the three given treatment groups, the second littermate was then assigned to the "matched" control arm, and so forth to obtain the highest possible degree of consistency and to avoid randomization bias as far as possible. All mice were treated until they developed abdominal distension, reflecting the accumulation of hemorrhagic ascites, typically occurring within 48 to 72 hours before death [23].

Histologic Evaluation

After completion of drug treatment, mice were euthanized, blood was collected from the thoracic cavity for serum analysis, and pancreas and liver were removed and inspected for grossly visible tumors and metastases, and both were preserved in 10% formalin solution (Sigma-Aldrich) for histology and processed for RNA extraction (see below). Formalin-fixed, paraffin-embedded tissues were sectioned (4 μm) and stained with hematoxylin and eosin (H&E). Six sections (100 μm apart) of pancreatic and liver tissues were histologically evaluated. *LSL-Kras^{G12D};LSL-Trp53^{R172H};Pdx1-Cre* mice were classified by having developed invasive pancreatic cancer or not.

Immunostaining

For immunolabeling, formalin-fixed and paraffin-embedded archived tumor samples and corresponding normal tissues were stained as previously described [22]. Concentrations and sources of primary antibodies are available on request. Briefly, slides were heated to 60°C for 1 hour, deparaffinized using xylene, and hydrated by a graded series of ethanol washes. Antigen retrieval was accomplished by microwave heating in 10-mM sodium citrate buffer, pH 6.0, for 10 minutes. For immunohistochemistry, endogenous peroxidase activity was quenched by 10-minute incubation in 3% H_2O_2. Nonspecific binding was blocked with 10% serum. Sections were then incubated with primary antibodies overnight at 4°C. For immunohistochemistry, bound antibodies were detected using the avidin–biotin complex (ABC) peroxidase method (ABC Elite Kit, Vector Labs, Burlingame, CA). Final staining was developed with the Sigma FAST DAB peroxidase substrate kit (Sigma, Deisenhofen, Germany). Masson's trichrome was used to visualize the extracellular matrix (blue) (10×). The immunohistochemistry results were scored as described previously [24]: negative = less than 5% cells positive; + = <30% cells positive; ++ = >30% cells positive. Positive cells were counted by manual assessment within defined 10× fields of view (n = 3/section; 3 sections analyzed/animal).