Telomerase Transcriptional Targeting of Inducible Bax/TRAIL Gene Therapy Improves Gemcitabine Treatment of Pancreatic Cancer

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Currently, gemcitabine is approved as the first-line therapy for patients with locally advanced or metastatic pancreatic cancer. Unfortunately, because of pre-existing or acquired chemoresistance of most of the tumor cells, gemcitabine has failed to significantly improve the outcome for pancreatic carcinoma patients. The present study explored the possibility of sensitizing pancreatic cancer to gemcitabine chemotherapy by combining the chemotherapy with the proapoptotic genes *Bax* and TNF-related apoptosis-inducing ligand (*TRAIL*). We designed two tetracycline-inducible recombinant adenoviruses using the human telomerase reverse transcriptase (hTERT) promoter for transcriptional apoptogene targeting. Our data showed that treatment with the adenoviral systems resulted in high-level expression of *Bax* and *TRAIL* genes directly related to apoptosis induction, leading to a significant sensitization of resistant pancreatic tumor cells. Furthermore, treatment with Bax and TRAIL adenoviruses plus a suboptimal dose of gemcitabine resulted in significant tumor regression and prolongation of the experimental animal's life, in contrast to the weak retardation in tumor growth observed when gemcitabine alone was used. Additionally, using an orthotopic tumor model, we showed the usefulness of a non-invasive whole-body optical imaging for real-time evaluation of therapeutic efficacy. Together, these findings suggest that hTERT-targeted proapoptotic gene expression in combination with gemcitabine may be a potential therapeutic strategy for treatment of pancreatic adenocarcinoma.

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INTRODUCTION

Human pancreatic cancer is a devastating disease characterized by a very poor prognosis. Today, it is the fourth leading cause of cancer-related death in the European Community and in North America.[1,2] Little is known about the causes of this disease which is difficult to diagnose in its early stages, and patients with inoperable pancreatic cancer have a dismal prognosis with a mean life expectancy of 3–6 months.[2,3]

For a long time, 5-fluorouracil was the most widely used chemotherapeutic agent in metastatic pancreatic cancer. However, it had only a modest effect and was associated with high systemic toxicity.[4,5] At present gemcitabine, a pyrimidine analogue of deoxycytidine with a wide range of antitumor activity against solid tumors, has become the standard of care for patients with locally advanced and metastatic pancreatic cancer.[6–8] In randomized trials, gemcitabine was the first and only chemotherapeutic agent that has been shown to have any meaningful impact on either survival or disease-related symptoms in pancreatic adenocarcinoma with few systemic side effects.[9] Although this drug improves the quality of life in many patients, it prolongs survival of pancreatic cancer patients by only a few months.[10] The reasons for the resistance of pancreatic cancer to therapy are not clearly understood, but the multidrug resistant mechanisms and the lack of response to apoptotic stimuli are widely recognized in cancer chemotherapy.[11] Thus, in spite of some progress having been achieved in the treatment of pancreatic cancer, the majority of the patients still succumb to cancer, and it is clear that better treatment strategies are urgently needed. The future of pancreatic cancer therapy may lie in the new fields of targeted and molecular therapies. Besides improvement of the conventional chemotherapy, cancer gene therapy is a particularly promising approach, and includes the use of suicide gene/prodrug systems, inhibition of oncogene expression, blocking of tumor angiogenesis, antitumor immuno-modulation, and tumor-specific apoptosis induction.

The failure of chemotherapy can be attributed, at the cellular level, to an acquired resistance to apoptosis. Therefore devising mechanisms to restore the apoptotic process in tumor cells may help in the design of effective therapeutic strategies against resistant cancers.[12,13] In the recent past, a wide variety of apoptosis-inducing molecules have been identified. Two major apoptotic pathways originating from separate subcellular compartments have been identified as (i) the death receptor–mediated "extrinsic apoptotic pathway" and (ii) the mitochondrion-mediated "intrinsic apoptotic pathway".[14,15] While each pathway is initially mediated by different mechanisms, they share a common final phase of apoptosis, consisting of activation of the executioner caspases and dismantling of the substrates that are critical for cell survival. Several studies separately using the proapoptotic genes, *Bax* and TNF-related apoptosis-inducing ligand (*TRAIL*), showed that their overexpression leads to apoptosis in a wide variety of tumor

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cell lines; these genes appear to be promising candidates for cancer therapeutics.[15,16] It is therefore rational to study the proapoptotic activity of TRAIL and Bax together in cancer cells.

On the basis of these data, and taking into account the generally favorable toxicity profile of gemcitabine in patients, we hypothesize that simultaneous overexpression of Bax and TRAIL-induced apoptosis pathways would elicit a stronger antitumor effect and chemosenzitisation of pancreatic cancer.

In order to prevent any apoptotic side-effect in normal cells, selective and targeted gene expression can be achieved through transcriptional regulation using tissue- or cell-specific promoter elements. Human telomerase reverse transcriptase (hTERT), the catalytic subunit of the telomerase, is virtually undetectable in most normal tissues[17] and is transcriptionally upregulated in more than 80% of tumor cells, including pancreatic cancer.[18,19] By reducing cytotoxicity in normal cells, the use of specific promoters provides a clear advantage over the strong constitutive promoters such as the human cytomegalovirus (CMV) and the phosphoglycerate kinase (PGK) promoters.

In the present study, we propose a single bicistronic adenoviral vector containing an internal ribosome entry segment (IRES) to express Bax-ires-GFP or TRAIL-ires-GFP under the control of hTERT promoter, using an inducible Gal4/GV16 fusion protein by modifying the Tet-Off system. The efficacy of these recombinant adenoviruses, named Ad-igBax and Ad-igTRAIL, was evaluated alone and in combination with gemcitabine, both *in vitro* and *in vivo* in pancreatic carcinoma models.

The results of the present study demonstrate that targeted overexpression of apoptosis-inducing *Bax* and *TRAIL* genes in combination with gemcitabine might provide an effective approach to pancreatic cancer therapy.

RESULTS

Construction of the recombinant adenoviruses Ad-igBax and Ad-igTRAIL

In order to reduce the adenoviral dose and to solve the problem of production and amplification of adenovirus-expressing toxic or apoptotic genes, we opted for a tetracycline-inducible system for Bax and TRAIL expression targeting, using the hTERT promoter. In addition, in order to facilitate the evaluation of the adenovirus function, *Bax* and *TRAIL* genes were co-expressed with green fluorescent protein (GFP), using an IRES element. We thus designed bicistronic tetracycline inducible adenoviruses named Ad-igBax and Ad-igTRAIL. On the basis of the Tet-Off system, the hTERT promoter drives the expression of a transactivator fusion protein TetR/VP16 (**Figure 1a**). In the absence of tetracycline, tTA binds to tetracycline-responsive element (TRE) and induces the expression of GFP, Bax, and TRAIL. The functionality of these adenovirus constructions was evaluated, and it was indicated that low concentrations of tetracycline were able to inhibit Bax expression. Indeed, at 0.1 µg/ml of tetracycline treatment, Bax expression was drastically inhibited; using 1 µg/ml of tetracycline the level was close to the endogenous Bax protein. Moreover, the tumor cells infected with Ad-igBax, without any treatment, showed a high Bax protein expression, comparable to that induced by the binary system combining Ad/PGK-GV16 and Ad/GT-Bax (**Figure 1b**).

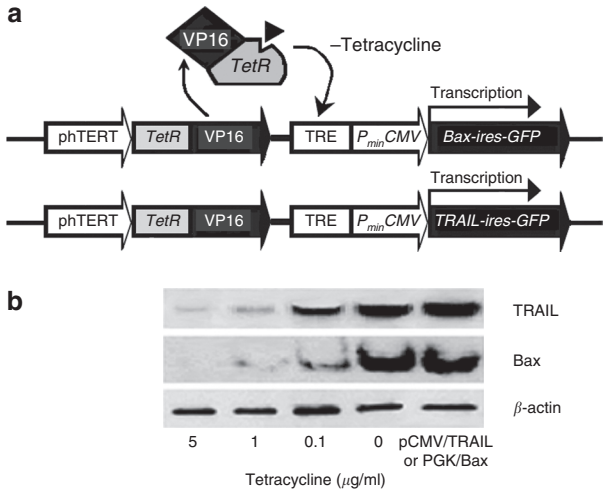

Figure 1 Construction of Ad-igBax and Ad-igTRAIL adenoviruses. (**a**) Schematic diagram of inducible recombinant adenoviruses (Ad-igBax and Ad-igTRAIL). Bax and TNF-related apoptosis-inducing ligand (TRAIL) are under the control of a chimeric promoter "tetracycline-responsive element (TRE) and cytomegalovirus (CMV) minimum promoter", which is silent unless activated by tTA (TetR/VP16). In the absence of tetracycline, tTA binds to TRE and induces the production of a bicistronic messenger RNA which, in the presence of an internal ribosome entry site (IRES), is translated into Bax or TRAIL and green fluorescent protein (GFP). (**b**) Tetracycline dependent-expression of Bax and TRAIL in Panc-1 cells transduced with Ad-igBax or with Ad-igTRAIL. Cells were grown in the absence or presence of increasing tetracycline concentrations for 24 hours, at the end of which total proteins were extracted and analyzed for Bax and TRAIL expressions. The addition of tetracycline (1 µg/ml) resulted in a potent inhibition of transgene expression.

Apoptosis-inducible gene expression is restricted to telomerase-positive tumor cells

In order to assess the transcriptional activity of the hTERT promoter, Panc-1, Capan-1, and fibroblast cells were infected with Ad-igTRAIL or Ad-igBax [multiplicity of infection (MOI) 50] in the presence or absence of hTERT-small interfering RNA (hTERT-siRNA) (100 nmol/l). Reverse transcriptase-polymerase chain reaction (RT-PCR) assay demonstrated that infected Panc-1 and Capan-1 cells yielded strong bands of the Bax and TRAIL transcripts 48 hours after infection, whereas the bands were extremely weak in siRNA hTERT-transfected cells and in fibroblasts infected with Ad-igBax or Ad-igTRAIL (**Figure 2a**). In order to further confirm the transcriptional activity, Western blot experiments were carried out using specific Bax and TRAIL antibodies. As shown in **Figure 2a**, Bax and TRAIL protein expressions were readily achieved only in positive cells 48 hours after adenovirus infection. In contrast, there was little, if any, detectable Bax and TRAIL expressions in fibroblasts and in hTERT siRNA-transfected cells.

Ad-igBax and Ad-igTRAIL induce overexpression of Bax and TRAIL

In order to confirm the functionality and efficiency of the designed adenoviruses Ad-igBax and Ad-igTRAIL, the expressions induced by them were compared with those induced by the binary system using the PGK ubiquitous strong promoter (Ad/PGK-GV16 and Ad/GT-Bax). As shown in **Figure 2b**, RT-PCR and Western

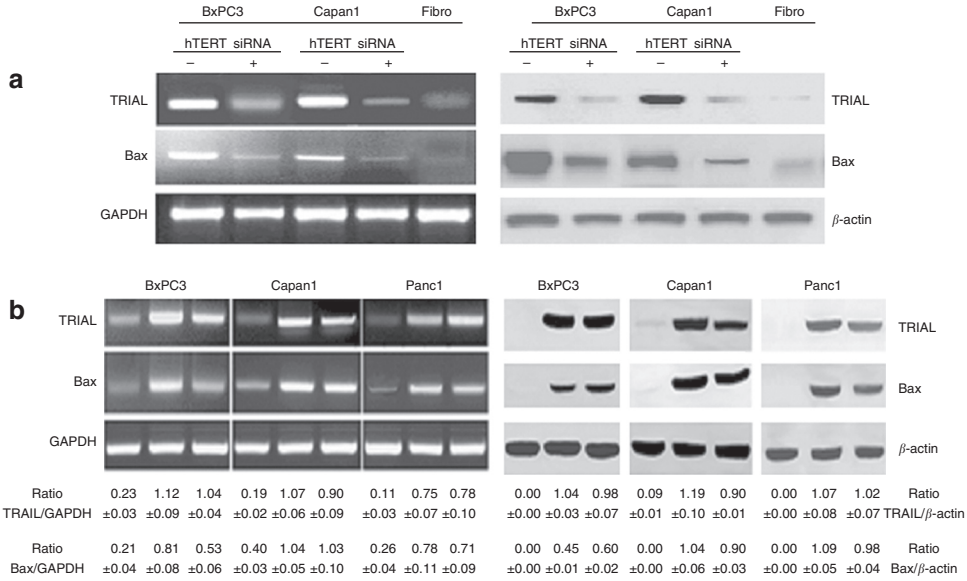

Figure 2 Efficacy and specificity of Bax and TNF-related apoptosis-inducing ligand (TRAIL) expression. (**a**) Telomerase-dependant expression of Bax and TRAIL was determined using telomerase-negative normal fibrobroblats (Fibro) and cell lines positive for pancreatic cancer (Capan-1 and Panc-1). The human telomerase reverse transcriptase (hTERT) small interfering RNAs (siRNAs) were used for silencing endogenous telomerase expression in positive cells. (**b**) Representative photographs of reverse transcriptase-polymerase chain reaction and Western blot showing expression of Bax and TRAIL induced by hTERT promoter (lane 3) in comparison to that induced by the strong constitutive promoter phosphoglycerate kinase (lane 2). Lane 1 corresponds to untransduced cells, and shows the endogenous Bax and TRAIL expression. The densitometric quantification of Bax and TRAIL messenger RNAs and proteins is expressed as arbitrary units of relative value and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin, respectively.

blot data showed that transduction of tumor cells by the adenoviruses bearing either strong promoter PGK or the tissue-specific promoter hTERT resulted in Bax and TRAIL-specific bands of equivalent intensity (in comparison with the constitutive gene glyceraldehyde 3-phosphate dehydrogenase), whereas mock infection resulted in low basal expression. These results provide evidence of the efficacy of the hTERT promoter used for generating Ad-igBax and Ad-igTRAIL.

In vitro assessment of the antitumor effect of Ad-igBax and Ad-igTRAIL

In order to evaluate the sensitivity of BxPC-3, Capan-1, and Panc-1 to the overexpressed Bax and TRAIL genes, cell cultures were infected with the inducible hTERT recombinant adenoviruses Ad-igBax and Ad-igTRAIL, alone (MOI of 50) or in combination (total MOIs of 100 and 200). As indicated in **Figure 3a**, cell transduction with either Bax or TRAIL adenovirus resulted in a significant decrease in cell viability. In the control cell cultures, Ad/CMV-GFP had no obvious effect on any of these cell lines. In contrast, there was a strong apoptopic effect on the tumor cell lines in response to Bax and TRAIL-expressing vectors. It is important to note that the cell death induced by Ad-igTRAIL was found to be more pronounced than that obtained with Ad-igBax, and their combination resulted in enhanced cytotoxicity. The Capan-1 and Panc-1 cell lines were more affected by the combined adenovirus infection. The combination of Ad-igBax and Ad-igTRAIL at a total MOI of 200 produced an apoptopic effect similar to that produced by an MOI of 100. These results demonstrate that a combination of adenoviruses mediating the overexpression of Bax and TRAIL significantly inhibits tumor cell growth.

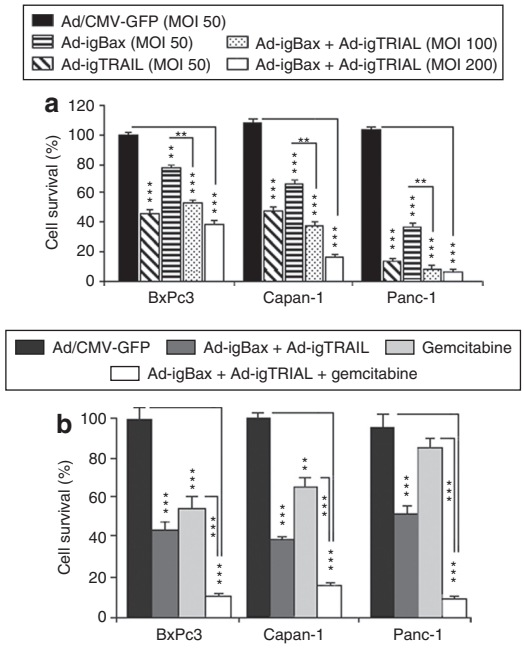

Figure 3 Enhanced antiproliferative effect of simultaneous Bax and TNF-related apoptosis-inducing ligand (TRAIL) expression and sensitization of human pancreatic cancer cells to gemcitabine. (**a**) Cell death induced by Bax and TRAIL alone or in combination, was determined 3 days after transduction, using MTT tests. Tumor cells infected with Ad/CMV-GFP were used as controls. (**b**) Simultaneous Bax and TRAIL expression sensitizes tumor cells to gemcitabine. Three days after treatment the percentage of cell survival was determined. The data are representative of three independent experiments (mean values ± s.e.m). Asterisks denote the difference between treated and untreated cells (100% cell survival). $^{**}P < 0.01$, ***significantly different from controls, $P < 0.001$. CMV, cytomegalovirus; GFP, green fluorescent protein; MOI, multiplicity of infection.

Enhanced efficacy of gemcitabine combined with Ad-igBax and Ad-igTRAIL

In order to determine whether a combination of Ad-igBax/Ad-igTRAIL with gemcitabine can inhibit the resistance mechanisms leading to a synergistic antitumor effect, we devised an experiment to analyze the sensitivity of various pancreatic tumor cell lines. Our data showed that the concentrations of gemcitabine required for 50% growth inhibition (IC_{50}) were 5×10^{8} mol/l, 10^{-7} mol/l, and 10^{-5} mol/l for BxPc3, Capan-1, and Panc-1, respectively (data not shown). We chose the concentration of 5×10^{-8} mol/l for further experiments in order to best observe any benefit of combination therapy. Subsequently, the tumor cells were treated with gemcitabine (5×10^{-8} mol/l) and Ad-igBax + Ad-igTRAIL (100 MOI), alone and in combination. As indicated in **Figure 3b**, our findings show clearly that the combined treatment resulted in a highly antiproliferative effect. More importantly, one can easily distinguish that the growth of the gemcitabine-resistant cell line Panc-1 was drastically inhibited. This Panc-1 tumor cell line was therefore selected for *in vivo* xenograft experiments.

Ad-igTRAIL plus Ad-igBax in combination with Gemcitabine induced a high rate of apoptosis

In order to confirm the results pertaining to cytotoxicity further, the Panc-1 tumor cell line was analyzed for apoptotic changes after treatment with Ad-igBax/Ad-igTRAIL and gemcitabine. After 3 days of treatment, Hoechst staining (**Figure 4a** and **b**) revealed a high chromatin condensation in the combined Bax/TRAIL-plus-gemcitabine treatment, and this was confirmed by the enzymatic terminal uridine deoxynucleotidyl transferase dUTP nick end labeling assay (**Figure 4c** and **d**). The DNA fragmentation was more pronounced after combined treatment using Ad-igTRAIL plus Ad-igBax plus gemcitabine (**Figure 4g**). We went on to examine whether the combined treatment would result in apoptosis of gemcitabine-resistant Panc-1 cells through activation of caspase-8, -9, and -3 (**Figure 4h** and **i**). Data from enzyme-linked immunosorbent assay measurements showed that induction of caspase-3 and caspase-9 appeared to be primarily caused by Ad-igBax + Ad-igTRAIL activity, with minimal contribution from the addition of gemcitabine. Only the activation of caspase-8 was significantly enhanced by gemcitabine co-treatment with Ad-igBax + Ad-igTRAIL ($P < 0.05$).

The hTERT promoter prevents toxic side effects *in vivo*

Finally, in order to assess liver toxicity in combined adenovirus or gemcitabine treatment, serum transaminase activity (glutamyl oxaloacetic transaminase, glutamyl pyruvic transaminase) was measured in mice after systemic and intratumoral administration of Ad-igBax or Ad-igTRAIL. Biological analysis showed that there was no significant elevation of serum transaminase levels in the animals. As regards glutamyl oxaloacetic transaminase and glutamyl pyruvic transaminase serum activity, no significant differences were seen between the groups receiving tumor therapy and an untreated control group. Gemcitabine, whether alone or in combination with adenoviruses, did not affect liver function (**Figure 5**). Additionally, the potential side effects in hematopoietic cells have been investigated, and the data clearly show that there were no significant changes in the values of hemoglobin, leukocytes, and platelets (data not shown).

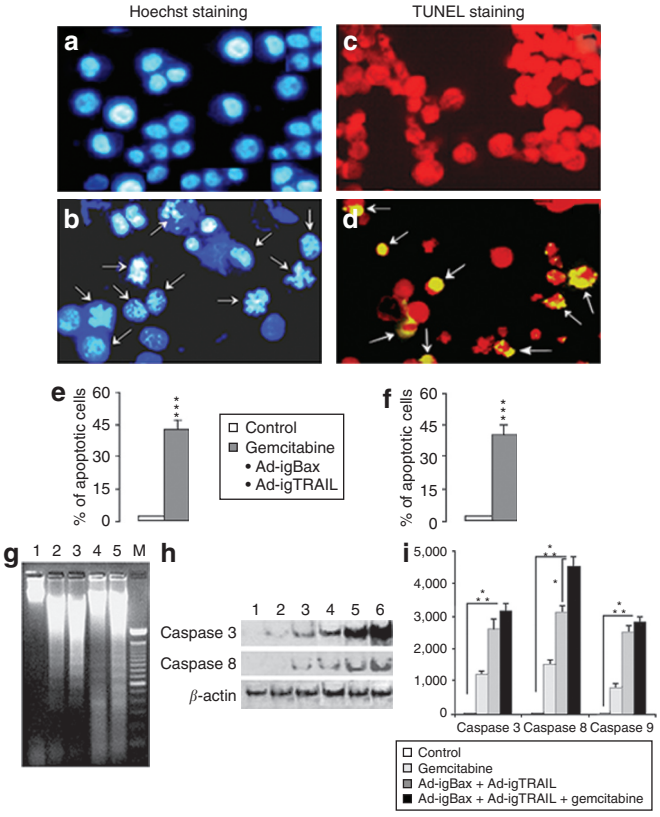

Figure 4 High apoptosis induction by gemcitabine combined with adenovirus-mediated Bax and TNF-related apoptosis-inducing ligand (TRAIL) expression. Three days after treatment, tumor cells (Panc-1) were fixed and subjected to (**a**, **b**) the DNA-binding dye Hoechst 33342 and (**c**, **d**) terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. The micrographs **a** and **c** are consistent with control (untreated) cells, whereas micrographs shown in **b** and **d** correspond to combined treatment (Ad-igBax + Ad-igTRAIL + gemcitabine). Representative fields are presented from three independent experiments. Arrows indicate numerous cells undergoing apoptosis, as recognized by a condensed nuclear chromatin after Hoechst blue staining (**c**) and yellow staining corresponding to dUTP-labeling of DNA strand breaks of apoptotic cells with TUNEL (**d**). Data from **a**, **b** and **c**, **d** were used for quantitative estimation; at least three independent experiments, each performed in triplicate, were carried out for each condition, and a minimum of 500 cells were counted for each measurement (**e, f**). (**g**) Seventy-two hours after treatment, DNA was extracted and analyzed for internucleosomal DNA fragmentation as described in Materials and Methods. Lane 1, untreated cells; Lane 2, gemcitabine; Lane 3, Ad-igBax; Lane 4, Ad-igTRAIL; and Lane 5, combined treatment (gemcitabine with Ad-igBax and Ad-igTRAIL). Lane M shows a 1 kb DNA marker. (**h**) Representative immunoblots and (**i**) enzyme-linked immunosorbent assay histograms were determined in order to evaluate apoptosis related to caspase activation. Lane 1, untreated cells; lane 2, gemcitabine; lane 3, Ad-igBax; lane 4, Ad-igTRAIL; and lane 5, Ad-igBax plus Ad- igTRAIL. Lane 6 corresponds to the tumor cells exposed to Ad-igBax and Ad-igTRAIL plus gemcitabine. Data are expressed in arbitrary units per μg of protein and represent the average values from four independent experiments, each performed in duplicate. The statistical analysis showed that differences between combination-treated and control cells were statistically significant (***$P < 0.001$).

In vivo antitumor activity of Ad-igBax, Ad-igTRAIL, and gemcitabine

We next carried out a series of *in vivo* experiments to confirm the *in vitro* observations and to determine whether the efficacy of Ad-igBax/Ad-igTRAIL against pancreatic adenocarcinoma xenografts could be enhanced by using it in combination with gemcitabine.

In order to evaluate adenovirus tumor transduction, we first investigated the levels of Bax gene expression at days 10 and 30 after treatment, using RT-PCR. In these experiments, we have compared our tetracycline-inducible Ad-igBax and Ad-igTRAIL with the binary system combining Ad/PGK-GV16 + Ad/GT-Bax, as described by Kagawa S. *et al.*[12] At day 10, Bax expression induced with Ad-igBax was significant and equivalent to that obtained with the non-specific PGK promoter. This expression was time-dependant and appeared to persist for at least 3 weeks. Indeed, 30 days after infection, Bax messenger RNAs and proteins were reduced to the base level, whereas in Ad/igBax-infected tumors it was sustained (**Figure 6a** and **b**).

In parallel, the tumor volumes were monitored for 7 weeks. As indicated in **Figure 6c**, tumors infected with Ad/CMV-GFP were not affected when compared with untreated tumors. However, gemcitabine reduced the tumor volume significantly ($P < 0.01$). More importantly, in the other treated groups, we observed that Ad-igBax or Ad-igTRAIL were able to reduce tumor growth significantly ($P < 0.001$), with the same efficacy as the adenovirus bearing a strong non-specific promoter does. The next experiments were focused on the antitumor efficacy of the combined treatment (inducible adenoviruses plus gemcitabine). As is shown in **Figure 6d**, the combination of Ad-igBax + Ad-igTRAIL with gemcitabine drastically reduced tumor growth. Together, these findings highlight the potential benefit of the combined therapeutic regimen using Bax/TRAIL with gemcitabine in the treatment of pancreatic tumors.

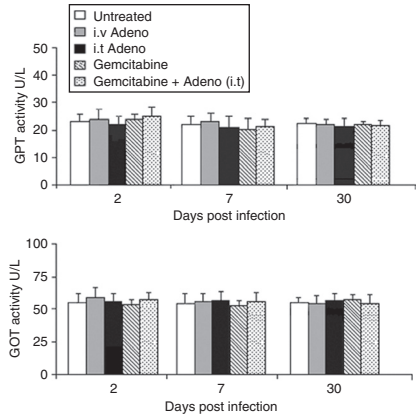

Figure 5 No obvious liver toxicity after administration of gemcitabine and replication-deficient recombinant adenoviruses. For analysis of liver function, serum samples were collected from live mice at days 2 and 10, and the glutamyl oxaloacetic transaminase (GPT) and glutamyl pyruvic transaminase (GOT) levels were monitored as indicated in Materials and Methods. The data represent the mean values for five animals; bars ± s.e.m.

In vivo tumor-specific targeting of Ad-igBax plus Ad-igTRAIL combined with gemcitabine

In order to obtain a clinical perspective, an experiment was carried out using mice bearing orthotopic pancreatic tumors "Panc-1/GFP". The animals were injected intraperitoneally three times

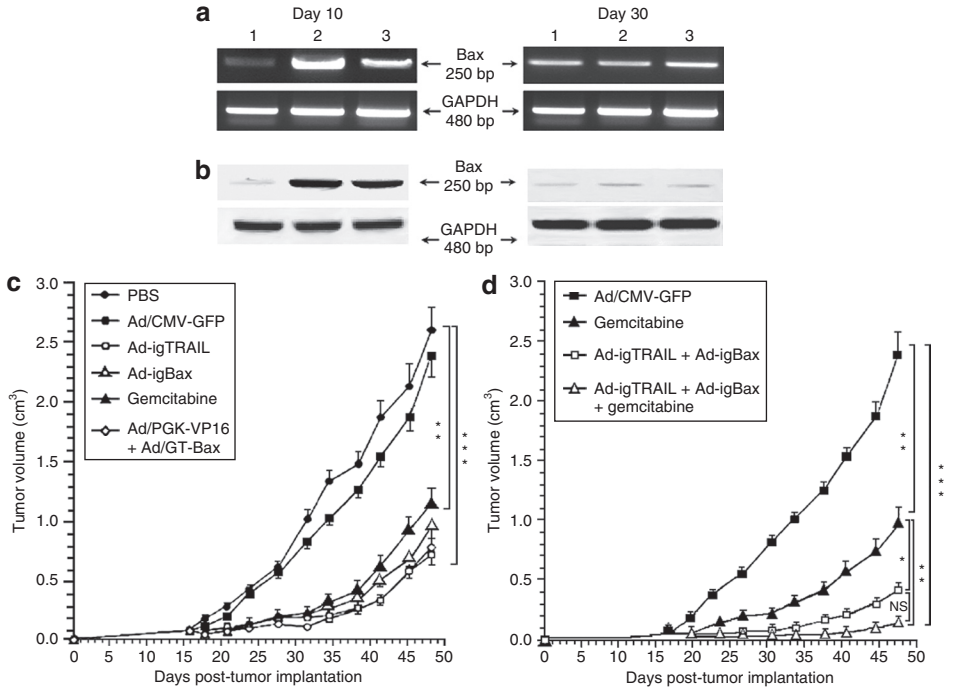

Figure 6 Transduction efficacy and tumor growth inhibition of subcutaneously (SC) xenografted pancreatic Panc-1 tumors. Panels (**a**) and (**b**) are, respectively, the representative reverse transcriptase-polymerase chain reaction and immunoblot data obtained from tumor samples collected at day 10 and day 30 after treatment. For assessing tumor growth, the comparative antitumor effects of (**c**) single and (**d**) combined treatment modalities were evaluated as described in Materials and Methods. Statistical differences in tumor sizes were evaluated at the end of the experiment (NS, non significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

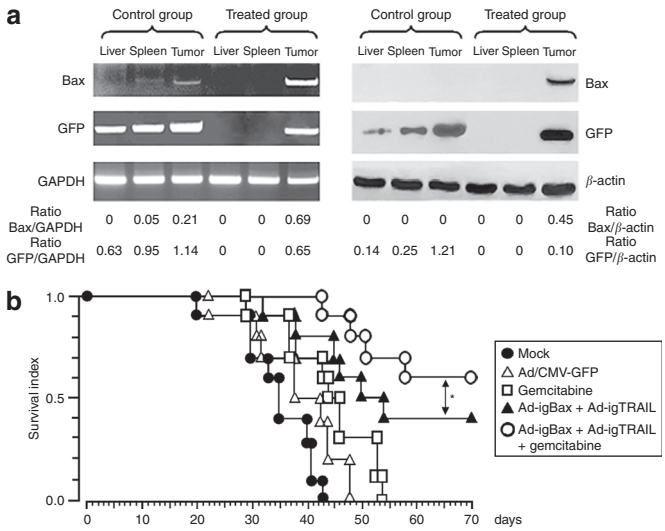

Figure 7 Prolongation of the survival time of mice with orthotopic pancreatic tumors (GFP-Panc-1) after specific apoptogene expression targeting and gemcitabine treatment. (**a**) Efficacy of tumor cell transduction and targeting of apoptogene expression were assessed 3 days after the last injection. Representative data from reverse transcriptase-polymerase chain reactionand Western blot, obtained with the corresponding biopsies from the control group (Ad/CMV-GFP) and the treated group (Ad-igBax). Green fluorescent protein (GFP) and Bax expression were compared with internal controls [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin]. (**b**) The survival rate of orthotopic tumor–bearing mice was monitored over time after treatment, and plotted as a Kaplan–Meier plot. The significance level versus mock was $P < 0.05$ for gemcitabine, $P < 0.01$ for Ad-igBax/Ad-igTRAIL, and $P < 0.001$ for Ad-igBax/Ad-igTRAIL plus gemcitabine. The differences between Ad-igBax/Ad-igTRAIL plus gemcitabine and Ad-igBax/Ad-igTRAIL treated groups were compared and considered significant ($P < 0.05$).

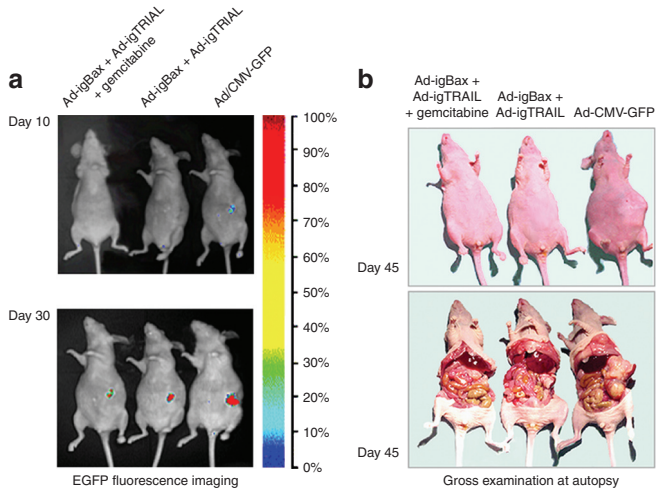

Figure 8 Noninvasive whole-body fluorescence imaging of suppression of orthotopic pancreatic tumor progression by TNF-related apoptosis-inducing ligand (TRAIL) and Bax apoptogene therapy in combination with gemcitabine. (**a**) Images show Panc-1 pancreatic tumor [expressing green fluorescent protein (GFP)] progression, and evaluation of therapeutic efficacy over time. The images were obtained using real-time whole-body imaging of representative mice from each group on days 10 and 30 after treatment. (**b**) Macroscopic aspects of mice and gross examination at autopsy of orthotopic intrapancreatic tumors at day 45 after tumor implantation. In contrast to mice treated with Ad-igBax/igTRAIL plus gemcitabine, those infected with Ad/CMV-GFP, show prominent swollen abdomens invaded by tumor nodules.

with Ad-igBax and Ad-igTRAIL. In order to evaluate gene targeting, Bax expression was investigated in liver, spleen, and tumor biopsies by RT-PCR and Western blot at days 10 and 30 after the last injection of adenoviruses. **Figure 7a** shows that the Bax gene is highly expressed in Ad-igBax-treated tumors. At the same time, it is worth noting that these mice showed a GFP expression restricted to the pancreatic tumor tissue, whereas in the control group this expression is present not only in the tumor, but also in the spleen and the liver. These results confirm the specific targeting and high expression of apoptotic gene driven by hTERT promoter.

According to Kaplan–Meier, all the untreated control tumor-bearing mice (Mock) and all those treated with Ad/CMV-GFP died within 43 and 48 days, respectively, as indicated in **Figure 7b**. Gemcitabine-treated mice died within 54 days (log Rank test, $P < 0.05$). However, 40% of the tumor-bearing mice treated with Ad-igBax plus Ad-igTRAIL were still alive after 2 months (log Rank test, $P < 0.001$), and a 60% survival rate was observed in the group receiving Ad-igBax/TRAIL plus gemcitabine (log Rank test, $P < 0.001$). At the autopsy, two-thirds of these surviving animals were tumor-free.

Real-time visualization of tumor growth and therapeutic efficacy evaluation

As our tumor model stably expresses GFP fluorescence, it enables real-time, sequential whole body imaging without the need for laparotomy, use of contrast agent for computerized tomography scan, or invasive procedures. The monitoring of the tumor growth assessed by optical imaging showed progressive time-dependant fluorescence (**Figure 8a**). The progression of disease in the control group (Ad/CMV-GFP) was rapid over the third and fourth weeks, leading ultimately to death in all the animals. On the contrary, after 1 month, the fluorescence in Bax/TRAIL-treated mice was significantly less than in AdCMV/GFP-treated mice. The fluorescence signal became minimally detectable when animals were co-treated with Ad-igBax/Ad-igTRAIL and gemcitabine. As shown in **Figure 8b**, tumor-bearing mice treated with Ad/CMV-GFP showed an increase of abdominal girth indicative of ascites and therefore tumor burden. These data were confirmed by autopsy examination. A very large tumor with locoregional invasion of vital structures was present in an Ad/GFP-treated mouse as compared to the very small tumor in a mouse receiving combined treatment.

DISCUSSION

Gemcitabine-based chemotherapy is considered the standard of care for most patients with locally advanced pancreatic cancer.[20–22] However, clinical reports have indicated the development of chemoresistance in gemcitabine-treated patients.[23] Strategies for overcoming resistance are essential to the success of anticancer therapy. Several studies have reported that resistance to apoptosis controls the ability of tumors to withstand high levels of chemotherapy.[24] Therefore, the development of approaches to enhance the apoptotic threshold within tumor cells is likely to prove useful both as a direct cancer treatment and in combination with chemotherapy. *TRAIL* and *Bax* are two

of the well-characterized proapoptotic genes, and their overexpression leads to apoptosis in a wide variety of malignant cells, with or without additional stimuli.[25,26] For a long time, apoptotic genes were speculated to be toxic for normal tissues. One common approach to target transgene expression is to use tissue- or cell-specific promoters,[27] one of which is the catalytic subunit of telomerase, hTERT. Indeed, it has been reported that telomerase activity is significantly higher in approximately 90% of cancers, and correlates well with the degree of malignancy. We therefore designed inducible tetracycline-recombinant adenoviruses, using the hTERT promoter sequence to target Bax and TRAIL gene expression. Additionally, we deliberately opted to use the Tet-Off system for two reasons (i) to directly control the production and amplification of the proapoptotic adenoviruses, and (ii) so that the tetracycline product could serve as an antidote in the case of any apparent cytotoxicity *in vivo*. In order to track the recombinant vector (and, indirectly, the proapoptotic gene *in vivo*) we constructed a bicistronic vector including a GFP with IRES.

In order to assess the antitumor activity, Ad-igBax and Ad-igTRAIL were used alone or in combination with gemcitabine. Our data showed that gemcitabine sensitized pancreatic cancer cells to Bax/TRAIL-mediated apoptosis. The sensitization can be obtained at subtoxic concentrations of gemcitabine and for a short duration of treatment. The combined treatment (Ad-igBax/Ad-igTRAIL with gemcitabine) drastically reduced tumor growth in the subcutaneous tumor model. Even if this result is not statistically significant, there is an unmistakable indication of reduction in tumor volume after combination therapy. We think that higher doses of the recombinant adenoviruses should be used for producing a more pronounced antitumor effect. In an experiment applicable to a clinic setting, we used an orthotopic pancreatic cancer model to demonstrate that combining Ad-igBax/Ad-igTRAIL with gemcitabine not only prolonged survival (almost two-thirds of the animals were still alive after 2 months), but also rendered 40% of the mice tumor-free at autopsy. The broad range of MOIs used for infecting cells and delivering transgenes in various gene therapy studies reflects both the lack of consensus among investigators for standardization of adenovirus vector concentrations and the fact that MOIs, especially in *ex vivo* and *in vivo* experiments, are estimated values based on arbitrary assumptions. In the present work, based on the commonly used convention of assuming approximately 10^8 cells per 1 cm^3 tumor, the significant *in vivo* antitumor efficacy and improved survival following the relatively low doses of Ad-igBax and Ad-igTRAIL (2×10^9 viral particles per dose) support the use of this safe and efficient apoptogene therapy protocol combined with gemcitabine. Furthermore, there was no detectable hepatotoxicity, and normal tissues from various organs were not affected by these treatments. In contrast, Ad/CMV-GFP generated expression in tumor but also in normal tissues such as liver and spleen of control mice. When mice were treated with Ad-igBax, significant GFP and Bax expressions were limited solely to the pancreatic tumor. This shows that hTERT promoter restricts the proapoptotic gene expression to tumor cell tissue, as corroborated by the apparent absence of liver toxicity. These findings show a synergistic tumor cytotoxic activity of Bax and TRAIL in combination with gemcitabine, thereby supporting the use of this approach in drug-resistant pancreatic cancer therapy.

Fluorescence molecular imaging systems allow follow-up of tumor development and growth in live animals.[28–31] Molecular imaging protocols can achieve these goals in real-time, non-invasively, rapidly, quantitatively, and repetitively in the same animal, under different conditions and stimuli. Moreover, this optical imaging strategy can improve the accuracy and efficiency of therapeutic schedules and avoid treatment remission. In the present study, the assessment of the antitumor activity was evaluated by caliper measurements of subcutaneous tumor model. This method is the most popular technique for cancer drug evaluation. However, it is not convenient for the increasingly used orthotopic or transgenic systems. Non-invasive imaging is ideally suited for follow-up studies on tumor growth and gene expression. Our results obtained with the orthotopic pancreatic cancer model confirm the antitumor effect of the combined treatment protocol (Ad-igBax/Ad-igTRAIL + gemcitabine). Up to day 7, no fluorescence signal was detectable in any of the mice that were examined. From day 10, molecular imaging could distinguish the control group from the others by a direct visualization of the tumors, which were almost invisible in the treated groups. These results were clearly in accordance with the laparatomy examination and with survival curve determination.

In this regard, establishing the correct therapeutic "window" of application for each tumor growth inhibitor (such as gene therapy intervention), or for drug combination, will be crucial in the management of pancreatic cancer treatment. The proof of this concept was elegantly demonstrated using optical imaging technology to follow up the efficacy of apoptogene therapy in combination with chemotherapy in an orthotopic pancreatic cancer model.

To summarize: on the one hand we provide solid evidence that apoptogene therapy targeting Bax and TRAIL combined with an optimized gemcitabine protocol constitutes a promising new approach to pancreatic cancer treatment and, on the other hand, we corroborate the usefulness of molecular imaging, which facilitates and accelerates the evaluation of the therapeutic efficacy of new anticancer strategies. As most of the patients will continue to present with unresectable pancreatic cancer, it is clear that the most effective therapy will require a combined approach incorporating the best-targeted interventions. Presently, we have combined conventional and new therapeutic approaches. The results suggest that multimodality therapy regimens using targeted suicide-apoptogene therapy that can overcome chemoresistance will provide new promise for pancreatic cancer treatment in the future.

Taking into account the data from this study and others, we believe that the concept of treating all patients with the same protocols will be given up by oncologists in the near future, and we are convinced that multimodal therapy is needed. It is crucial that we move forward with scientifically driven innovative therapies, as the empirical approaches have failed.

MATERIALS AND METHODS

Cell cultures and reagents. Human pancreatic cancer lines (BxPC-3, Capan-1, Panc-1, Panc-1/GFP), fibroblasts, and HEK293 cell line were

grown in Roswell Park Memorial Institute 1640 or in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37 °C in 5% CO_2. The Panc-1/GFP cell line was stably transfected with the plasmid EGFP-N1. Cell culture reagents and RT-PCR products were purchased from Invitrogen (Cergy Pontoise, France). Gemcitabine was obtained from Lilly (Fegersheim, Strasbourg, France).

Recombinant adenoviral vector construction and amplification. The construction of inducible and tumor-specific non-replicative adenoviruses expressing Bax or TRAIL was based on the AdenoX Tet-Off expression system (Ozyme; Clontech, Saint Quintin en Yvelines, France). Briefly, the complementary DNA corresponding to Bax or TRAIL was subcloned in pIRES2-GFP (Ozyme). The hTERT promoter sequence (−378/+78) was recovered from pGL3/hTERT-Luc provided by Inoue Masaki (Kanazawa University, Ishikawa, Japan) and substituted for the CMV promoter of pTet-off vector. Thereafter two cassettes were inserted in the pTRE-shuttle vector. The first cassette corresponded to Bax/ires/GFP or TRAIL/ires/GFP whose expression is under the control of a synthetic minimal promoter composed of TREs and a CMV minimum-promoter. The second cassette, with the transactivator tTA (tetR/VP16) driven by hTERT promoter, was inserted in the restriction site *Mfe*I of pTRE-shuttle. The fragment containing the entire two cassettes was excised from the pTRE-Shuttle vector by *I-CeuI* and *PI-SceI* and ligated unidirectionaly to AdenoX viral (*I-CeuI* and *PI-SceI* digested). The resulting inducible recombinant adenoviruses were named Ad-igBax (for Ad/hTERT-Bax/ires/GFP) and Ad-igTRAIL (for Ad/hTERT-TRAIL/ires/GFP). The binary system, Ad/PGK-GV16 and Ad/GT-Bax has been described elsewhere.[32] Recombinant viruses were propagated in HEK293 cells, purified and titered and stored as previously described.[33,34]

Functionality of recombinant adenoviruses.
RT-PCR: Total RNA was isolated using TRizol reagent and subjected to RNase-free DNase treatment. For complementary DNA synthesis, RNA (5 µg) was reverse-transcribed using random hexamer primers and superscript reverse transcriptase II (Invitrogen). PCR amplification was performed using specific primers for each target gene: Bax, forward 5′-TGCTTCAGGGTTTCATCCAGG-3′, reverse 5′-TGGCAAA GTAGAAAAGGGCGA-3′; TRAIL, forward 5′-GTACTCCAAAAGTG GCATT-3′, reverse 5′-CCATTTGTTTGTCGTT CTT-3′; and glyceraldehyde 3-phosphate dehydrogenase, forward 5′-ACCACAGTCCATGCC ATCAC-3′, reverse 5′-TCCACC ACCCTGTTGCTGTA-3′. The PCR conditions were: initial denaturation step at 94 °C for 3 minutes, followed by 35 cycles (Bax, TRAIL) or 30 cycles (glyceraldehyde 3-phosphate dehydrogenase) at 94 °C for 30 seconds, 58 °C (Bax, TRAIL) or 60 °C (glyceraldehyde 3-phosphate dehydrogenase) for 60 seconds, 72 °C for 60 seconds, and a final extension step at 72 °C for 7 minutes. PCR products were run on 1.5% agarose gels containing ethidium bromide.

Western blot analysis: Equal amounts of cell lysate protein (50 µg) were run on 4–12% NuPage Bis–Tris gradient gels (Invitrogen). Thereafter, proteins were transferred to a polyvinylidene difluoride membrane and blots were blocked with 5% bovine serum albumin in Tris buffer saline Tween-20 and incubated with appropriate primary antibody diluted in Tris buffer saline Tween-20 containing 5% non-fat milk (vol/wt) overnight at 4 °C. The mouse antibody against β-actin, diluted 1:1,000 (Sigma-Aldrich), was used as a control for equal gel loading. Immunoreactivity was detected using horseradish peroxidase–conjugated secondary antibody followed by enhanced chemoluminescence ECL Plus (Amersham Biosciences, Saclay, France).

Conditional apoptotic gene expression by hTERT promoter: In order to confirm that Bax and TRAIL are expressed only in telomerase-positive cells, normal human fibroblast and tumor cells were infected with Ad-igTRAIL or Ad-igBax (MOI 50) and the expression of apoptotic genes was analyzed 48 hours after infection by RT-PCR and Western blotting. The telomerase-dependent expression in tumor cells was confirmed using hTERT siRNA (Tébu-bio, Le Perray, En Yvelines, France). The telomerase-positive cells, Panc-1 and Capan-1, were co-treated with Ad-igBax or Ad-igTRAIL in combination with hTERT siRNA (100 nmol/l).

Growth inhibition assay: Cell viability was determined using the MTT test. Briefly, tumor cells were plated on 24-well plates one day prior to virus infection and/or gemcitabine treatment. Tumor cells with ~80% of confluence were infected with recombinant adenoviruses at various MOIs and incubated for a further 72 hours. In order to evaluate gemcitabine-induced cytotoxicity, cell cultures were treated with increasing concentrations of gemcitabine during 2 hours and then cultured for 3 days in a drug-free medium. The concentration of virus or gemcitabine required to inhibit proliferation by 50% (IC_{50}) was calculated from these results. Cell viability was expressed as the percentage of growth of untreated cells.

Apoptosis assessment.
Hoechst and terminal uridine deoxynucleotidyl transferase dUTP nick end labeling staining: Cells were plated on 15 mm diameter sterile Thermanox coverslips in 24-well plates. After treatment, fixed cells with 4% paraformaldehyde were incubated with 50 ng/mg Hoechst 33342 for 30 minutes at room temperature. For terminal uridine deoxynucleotidyl transferase dUTP nick end labeling labeling, apoptotic cells were detected using an ApopTag fluorescein direct *in situ* apoptosis detection kit (Qbiogene, Illkirch, France). For quantification, a minimum of 500 cells was counted from more than three random microscopic fields and the ratio of apoptotic cells was calculated as a percentage of total cells counted.

Genomic DNA fragmentation: In order to extend the cell morphology analysis, we performed experiments to examine the internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis. After treatment, floating and adherent tumor cells were collected and lysed for genomic DNA extraction and electrophoresis as described previously.[35]

Caspase expression and activity assay: After treatment, the cells were washed with phosphate-buffered saline and lysed in lysis solution, and the activity levels of caspase-3, -8, or -9 was determined using specific caspase colorimetric assay kits (Euromedex, Strasbourg, France). The expressions of caspase-3 and -8 were evaluated by Western blot analysis as described earlier (see Western blot section).

Pancreatic tumor models and treatment.
Animals: 7-8-week-old female athymic NMRI-nu (nu/nu) mice were purchased from Elevage Janvier, le Genest, France, and experiments were performed in accordance with the guidelines for use of laboratory animals (Ministère de l'Agriculture, France).

Subcutaneous pancreatic tumor model: Tumor xenografts were established by subcutaneous inoculation of 1.5×10^7 Panc-1 cells. When the tumors reached an average volume of 50–70 mm^3, two experiments were carried out. In the first experiment, the mice were divided randomly into six groups ($n = 10$): phosphate-buffered saline, Ad/CMV-GFP, Ad/PGK-VP16+Ad/GT, Ad-igBax, Ad-igTRAIL, and gemcitabine. The second experiment was designed to evaluate the antitumor efficacy of combined adenoviruses (Ad-igBax and Ad-igTRAIL), alone and with gemcitabine. Each mouse received three doses of intratumoral injections of phosphate-buffered saline or recombinant adenovirus (10^9 viral particles) on days 1, 4, and 7. Gemcitabine was injected intraperitoneally at 15 mg/kg/day, three times weekly for 2 weeks. For transgene expression analysis, two mice were killed 10 and 30 days after the last injection, and the tumor biopsies were snap frozen in liquid nitrogen and stored at −70 °C. Tumor volumes were monitored two times weekly and calculated (V = width × length × height × $\pi/6$).

Surgical orthotopic implantation of Panc-1/GFP tumors:. The mice were anesthetized by isoflurane inhalation and a small left abdominal flank incision was made; the pancreatic tail and the spleen were carefully exposed under aseptic conditions. The tumor Panc-1/GFP cells (1.5×10^7cells/50 µl) were injected into the exteriorized pancreatic corpus and the incision was closed in two layers. Two weeks later, the mice were randomized into

five groups (n = 14): Mock, Ad/CMV-GFP, gemcitabine, Ad-igBax + Ad-igTRAIL, and Ad-igBax + Ad-igTRAIL + gemcitabine. Recombinant adenoviruses (10^9 viral particles/200 µl) were administrated percutaneously in the pancreatic tumor area. Injections were repeated three times (days 1, 4, and 7). Gemcitabine (15 mg/kg) was administrated three times weekly for 2 weeks. Three days after the third infection, two mice from each group were killed, and biopsies from tumor, liver, spleen, mesenteric, and pancreas were excised and immediately snap frozen in liquid nitrogen. In the rest of the mice, the orthotopic tumor growth was followed by real-time whole-body optical imaging system (Hamamatsu, Massy, France), and the animals were monitored for health and survival. Mice were killed when they became moribund or exhibited lethargy and reduced mobility.

Assessment of liver damage and hematopoietic cell toxicity: After the tumor therapy with Ad-igBax, Ad-igTRAIL, and gemcitabine, blood samples were collected from the tail veins of the mice on days 1 and 10 so as to monitor liver damage, specifically, the serum liver enzyme levels of alanine transaminase and aspartate transaminase. For assessment of hematopoietic cell toxicity, aliquots of whole blood samples were taken and measured for the content of hemoglobin, leukocytes, and platelets. In order to simulate systemic adenoviral infection, we included a group of five mice that received intravenous injections of 10^9 plaque forming units Ad-igTRAIL or Ad-igBax instead of intratumoral injections.

Statistical analysis: The mean values ± s.e.m. were calculated for each data point. Differences between groups were analyzed by one- or two-way analysis of variance. Differences in the rates of complete tumor inhibitions or survivors were validated by student unpaired t-test. For synergistic or additive effects achieved after combined treatment, isobologram analysis was performed using the CalcuSyn software program, and a combination index of <1.0 indicates synergism.[36,37]

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