Advances in Brief

Therapeutic Efficacy of E2F1 in Pancreatic Cancer Correlates with *TP73* Induction[1]

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Abstract

Pancreatic cancer is particularly resistant to apoptosis by antineoplastic agents, which is partly attributable to the lack of functional p53. Here we show that E2F1 in combination with the most clinically efficient drug, gemcitabine, resulted in a strong induction of apoptosis independent of functional p53, whereas the effect of either therapy alone varied between different cell lines. Intratumoral injection of a helper-dependent adenovirus vector expressing E2F1 plus drug treatment resulted in a significant reduction of tumor volume. The therapeutic effect is directly correlated with the induction of the p53 homologue p73, suggesting that the recently discovered E2F1/p73 pathway plays a critical role in cancer therapy.

Introduction

Ductal pancreatic adenocarcinoma represents 80–85% of all of the pancreatic malignances and is one of the most lethal cancers in the Western world. Despite recent advances in diagnostics, staging, and therapy in pancreatic carcinoma, significant improvements in long-term survival have not been realized. A critical determinant of the efficacy of antineoplastic therapy is the ability of malignant cells to undergo apoptosis in response to DNA damage induced by radiation or cytotoxic agents. As a cause of resistance to genotoxic stress, the lack of functional p53, which is mutated in ≤70% of pancreatic adenocarcinomas, has been proposed to be a component of the inhibition of apoptosis (1). Thus, modulation of the apoptotic pathway represents a logical target for therapeutic intervention.

Several studies indicated that the cellular transcription factor E2F1 promotes apoptosis in various systems independent of the endogenous p53 status (2, 3). This has led to the suggestion that E2F1 may act as a potent tumor suppressor by engaging apoptotic pathways to protect organisms from developing tumors. In the present study, we constructed an E2F1-expressing Ad[3] vector and investigated the therapeutic effect of E2F1 alone and in combination with gemcitabine (2′-deoxy-2′-difluorodeoxycytidine; Gemzar)-based chemotherapy. Gemcitabine, a deoxycytidine analogue with structural and metabolic similarities to cytarabine, is more effective than fluorouracil in reducing symptoms in pancreatic cancer patients and confers a small survival benefit, which currently makes it the first-line therapy for patients with advanced pancreatic cancer (4). Our data indicate that E2F1 in combination with gemcitabine is capable of efficient killing of pancreatic tumors. The therapeutic effect is directly correlated with the induction of the p53 homologue p73 suggesting that the recently discovered E2F1/p73 pathway is an important target for cancer treatment.

Materials and Methods

Cell Culture. Human pancreatic adenocarcinoma cell lines AsPC-1 (ATCC CRL 1682), Capan-1 (ATCC HTB 79), Capan-2 (ATCC HTB 80), and MZA (obtained from David I. Smith, Mayo Clinic, Rochester, MN) were maintained in DMEM supplemented with 10% FCS. Viruses were grown in 293 (Ad5 E1-transformed human embryonic kidney cells) and 293Cre4 cells, respectively, maintained in MEM F-11 with 10% fetal bovine serum. AdER-E2F1 has been described previously (2). Medium were supplemented with 2 mM L-glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin.

Drug Treatment. The nucleoside analogue gemcitabine (2′,2′-diflurodeoxycytidine; Eli Lilly Co., Indianapolis, IN) was dissolved in PBS. For drug treatment, cells were exposed to 10 μM of gemcitabine alone or in combination with AdER-E2F1 infection for 1–5 days.

Construction of HD Vectors. The HD vector HD-AdE2F1 is based on the plasmid pSTK120 (5). The E2F1 expression cassette contains the rabbit β-globin intron in front of the E2F1 cDNA under control of the human CMV promoter terminated by the BGH poly(A) signal and was constructed as follows: (*a*) the CMV promoter plus the rabbit β-globin intron was isolated from the pCMV-E2F-1 plasmid by *Xba*I/*Bam*HI digest and subcloned into pCR3.1 (Invitrogen, Karlsruhe, Germany); (*b*) the E2F1 cDNA digested from pCMV-E2F1 by *Bam*HI was inserted into the *Bam*HI site of pCR3.1 behind the rabbit β-globin intron; (*c*) the expression cassette was released by *Pme*I and subsequently cloned into the *Cla*I site of pPGKpurobpA in front of the BGH poly(A). The complete expression cassette was digested by *Xba*I/*Not*I and integrated into the *Swa*I site of pSTK120. The plasmid pSTK129 used to construct the control virus HD-AdGFPS has a 26.5-kb insert in the multiple cloning site of Bluescript KSII containing the left terminus of Ad5 (nucleotides 1–440), a 19,952-bp fragment of the human hypoxanthine-guanine phosphoribosyltransferase gene (nucleotides 1,777–21,729 in gb:humhprtb), a 6,545-bp fragment of the C346 cosmid (nucleotides 10,205–16,750 in gb:L31948), and the right terminus of Ad5 (nucleotides 35,818–35,935). The expression cassette for the membrane-anchored GFPS was isolated from the plasmid pCM-VEGFP-Spectrin by *Ase*I/*Afl*II digestion and cloned into the *Not*I site of pSTK129.

Amplification and Purification of the HD Viruses. Adenoviral constructs were cleaved by *Pme*I and transfected into 293Cre4 cells (6). Subsequently the cells were infected with lox-P helper virus AdLC8cluc. To increase the titer, vector lysates were passed through 293Cre4 cells several times (7). The final stock was harvested from ~1.5 × 10⁹ 293Cre4 cells, followed by CsCl buoyant density centrifugation. The concentration for the purified viral particles for HD-AdGFPS was 1×10^{12}/ml and for HD-AdE2F1 3×10^{12}/ml. The contamination of lox-P helper virus in the virus preparation was <0.01%. For analysis of viral DNA, DNA from cells infected with CsCl purified virions was purified by overnight digestion at 37°C in Pronase-SDS buffer [10 mM Tris HCl (pH 7.4), 10 mM EDTA, 1% SDS, and 1 mg/ml Pronase], phenol extraction, ethanol precipitation, and analyzed by agarose gel electrophoresis.

MTT Assay and Flow Cytometry. Cells seeded on 60-mm dishes at 60–80% confluence were infected with Ad for 30 min at 37°C. After infection, cells were additionally incubated in the absence or presence of 1 μM of 4-OHT. For MTT assay triplicate wells of each treatment were assayed for cell viability 72 h after infection by the CellTiter96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega). For flow cytometry analysis, cells were harvested 48 h after infection, fixed in 70% ethanol, and stained for DNA content with propidium iodide. Analysis was carried out (FACSVantage; Becton Dickinson) and
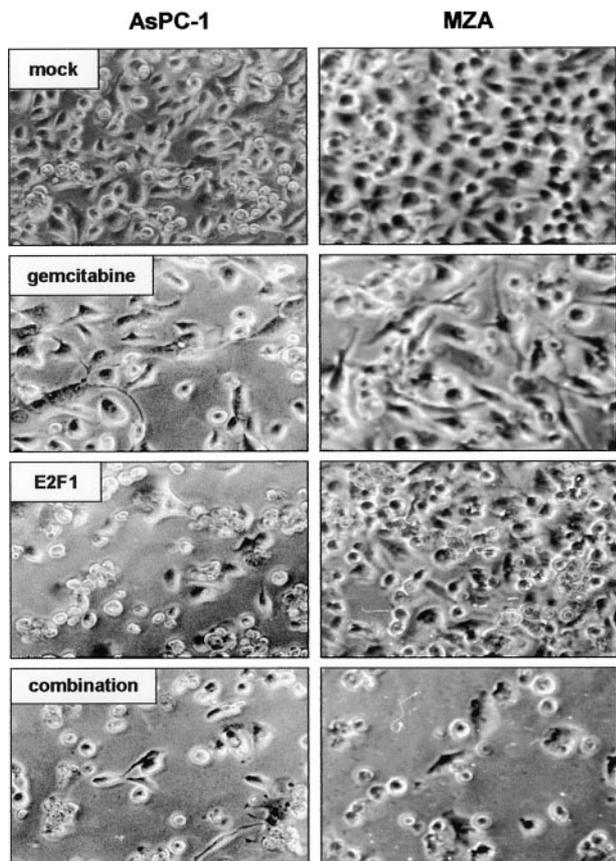
Fig. 1. Morphological changes induced by gemcitabine and/or AdER-E2F1 in pancreatic tumor cells. Shown are phase-contrast micrographs of AsPC-1 (*left*) and MZA cells (*right*) 48 h after treatment with gemcitabine, AdER-E2F1 in the presence of 4-OHT, or the combination compared with uninfected cells (*mock*).

analyzed as described previously using CellQuest software (Becton Dickinson).

Semiquantitative RT-PCR and Western Blotting. RT-PCR was performed on total RNA prepared by RNeasy Mini kit (Qiagen; Hilden, Germany) as described previously (8). The upstream and downstream primers used for the gene encoding p73 are 5′-GACGGAATTCACCACCATCCT-3′ and 5′-CCAGGCTCTCTTTCAGCTTCA-3′ and for glyceraldehyde-3-phosphate dehydrogenase 5′-CACAGTCCATGCCATCAC-3′and 5′-CACCACCCTGTT-GCTGTA-3′. For Western blot analysis cell lysates were prepared after infection, and protein levels were analyzed essentially as described (8) using monoclonal antihuman p53 (Ab-6; Calbiochem, Bad Soden, Germany) or antihuman E2F1 antibody (KH95; Santa Cruz Biotechnology, Heidelberg, Germany). Antibody binding sites were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Braunschweig, Germany).

Animal Experiments. Six- to eight-week-old *nu/nu* (nude) mice (Central Animal Facility, University of Essen, Essen, Germany) were s.c. injected into the right flank with 10^7 MZA tumor cells. Mice with visible tumors were injected intratumorally with the appropriate concentration of HD-AdE2F1 or the control vector HD-AdGFPS and monitored for tumor growth at twice weekly intervals. Tumors were measured in two perpendicular diameters using calipers. Animals were sacrificed when the longest diameter was >15 mm or when any two measurements were >10 mm.

Results and Discussion

E2F1 has been shown to trigger apoptosis in a variety of cell systems either alone or in association with p53 (3). To assess whether E2F1 gene expression affects the viability of pancreatic cancer cell lines, cells were infected with AdER-E2F1 at a multiplicity of infection, which allows 100% transduction. The AdER-E2F1 vector, in which transgene-mediated toxicity is regulated by fusion to the tamoxifen-inducible estrogen receptor ligand-binding domain, has recently been shown to be efficient in the induction of cell death in tumor cells lacking functional p53 (2). With E2F1 alone, we observed differences in the cell viability between the various cell lines (Fig. 1 and Fig. 2*A*) with an apoptotic rate ranging from 5.81% in MZA to 23.59% in AsPC-1 cells (Fig. 2*B*). Next, we analyzed the effect of E2F1 in combination with gemcitabine, the current first-line therapy for pancreatic cancer patients with advanced disease. On the basis of previous studies, it is known that the cytotoxicity of gemcitabine strongly correlates with the amount of drug incorporated into the cellular DNA up to the maximum achievable intracellular concentration of 20 μM (9, 10). Therefore, the therapeutic efficacy of gemcitabine was analyzed in various pancreatic cancer cell lines using gemcitabine at a clinically relevant dose of 10 μM. We observed comparable levels of cytotoxicity by gemcitabine treatment alone with slight variations between the different cell lines (Figs. 1 and 2). Importantly, all of the cell lines tested exhibited a profound loss of cell viability on combination therapy of E2F1 and gemcitabine (Fig. 2*A*) with morphological changes characteristic for cells undergoing apoptosis as shown for MZA and AsPC-1 cells by phase contrast micros-

Fig. 2. Analysis of cell viability and apoptosis in human pancreatic cancer cell lines. *A,* cell viability of MZA, AsPC-1, Capan-1, and Capan-2 cells 72 h after treatment with gemcitabine (*dark gray bar*), AdER-E2F1 in the presence of 4-OHT (▨), and the combination of AdER-E2F1 plus drug (□) as measured by MTT assay. Cell viability of untreated cells (■) was set as 100%. The number of viable cells is the mean ± SD of three different wells; *bars,* ± SD. Triplicate experiments were performed for each cell line. *B,* flow cytometry DNA profiles of AsPC-1 (*left*) and MZA cells (*right*) 48 h after treatment with mock (*I*), gemcitabine (*II*), AdER-E2F1 in the absence (*III*) and presence of 4-OHT (*IV*), and the combination of AdER-E2F1 plus drug (*V*). Apoptotic cell population is labeled as M1.

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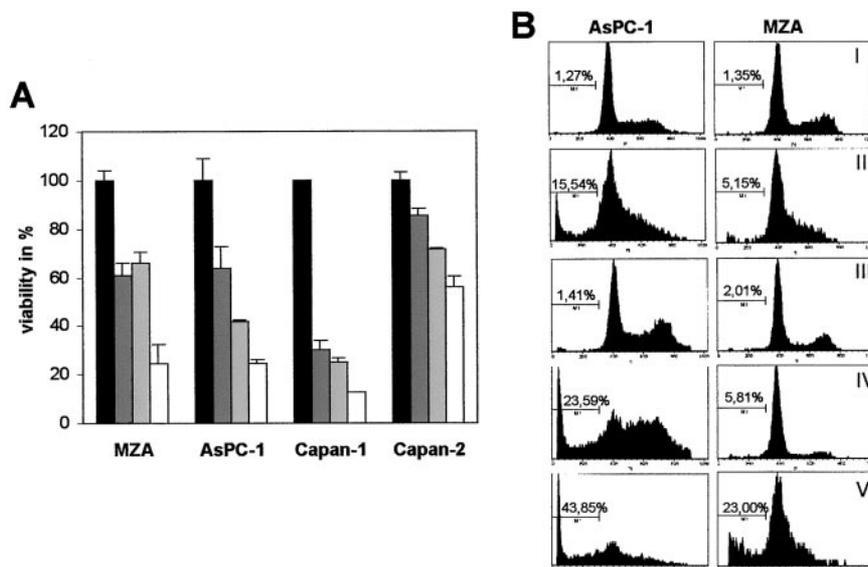
copy (Fig. 1) and Hoechst-33342 staining (data not shown). Flow cytometry analysis of both cell lines treated with AdER-E2F1 plus drug clearly revealed a significant increase of sub-G_1 cells (Fig. 2*B, panel V*) indicative of apoptosis. Although E2F1 alone had almost no effect in MZA cells it clearly sensitized to gemcitabine-induced apoptosis resulting in 23% apoptotic cells (Fig. 2*B, panel V*). In contrast, no increase in the sub-G_1 population appeared in untreated controls (Fig. 2*B, panel I*) and AdER-E2F1-infected tumor cells in the absence of ligand (Fig. 2*B, panel III*). These data indicate that E2F1-mediated killing of pancreatic cancer cells can be efficiently enhanced by gemcitabine treatment.

One mechanism by which E2F1 induces apoptosis relies on the activation of the *CDKN2A* transcript p14ARF, resulting in p53-dependent apoptosis (11). We have shown recently that part of the apoptotic activity of E2F1 reflects the ability to induce expression of the p53 homologue p73 by direct binding to the *TP73* promoter (8). In line with the frequent inactivation of p53 in pancreatic cancer, we did not observe an increase in p53 protein levels after E2F1 expression in MZA cells, suggesting that activation of the p53 pathway is not involved in E2F1-mediated apoptosis in both MZA and p53-negative AsPC-1 cells. In contrast, induction of *TP73* directly correlated with the apoptotic activity of E2F1 (Fig. 3). In AsPC-1 cells, E2F1 alone is sufficient to induce *TP73* expression and apoptosis, whereas in MZA cells this effect is only achieved by the combination treatment, suggesting that activation of *TP73* is required for efficient induction of apoptosis as a prerequisite for tumor growth suppression *in vivo*. At present, it is not clear why *TP73* is not induced by E2F1 alone in MZA cells. Because E2F1 protein levels are not affected by gemcitabine treatment, lack of *TP73* activation may be attributable to inhibition of E2F1, for example, by high levels of p14ARF, which have been shown to inhibit E2F1 transactivation (12). The effect of gemcitabine on E2F1-mediated transactivation is currently under investigation.

In the context of our *in vitro* data, we analyzed the therapeutic efficacy of combination therapy in mice. To avoid vector-related toxicity, established s.c. tumors were treated by a single intratumoral injection of 3×10^{12} particles of a HD E2F1 expressing Ad-vector (HD-AdE2F1; Fig. 4*A*) combined with i.p. gemcitabine injections (60 mg/kg) over 10 days (days 1, 4, 7, and 10). Tumors were monitored for regression in comparison with treatment with only gemcitabine, HD-AdE2F1, or the HD-AdGFPS control virus. Both HD-AdE2F1 or drug alone showed a similar antitumoral effect, resulting in a delayed

Fig. 4. Antitumor activity of HD-AdE2F1/gemcitabine combination treatment on established pancreatic tumors. *A*, structure of HD-AdE2F1. The E2F1 expression cassette contains the rabbit β-globin intron in front of the E2F1 cDNA under control of the human CMV promoter terminated by the BGH poly(A) signal as described in "Materials and Methods." *B*, mean tumor volume of mice measured every 3–5 days; *C*, long-term survival of the experimental animals plotted as a function of time. ♦, HD-AdGFPS ($n = 5$); ■, gemcitabine ($n = 5$). ▲, HD-AdE2F1 ($n = 5$); ✕, HD-AdE2F1+ gemcitabine ($n = 5$). Tumors were treated either by multiple i.p. injections of gemcitabine (60 mg/kg) over 10 days (on days 1, 4, 7, and 10), direct injection with HD-AdE2F1 (3×10^{12} particles), or the combination of gemcitabine and HD-AdE2F1. HD-AdGFPS was used as a control.

tumor growth compared with control vector-injected animals but failed to prevent tumor progression (Fig. 4*B*). A pronounced effect on tumor growth was observed only after combination treatment, which caused a substantial retardation of tumor growth over ≥25 days compared with mice, which received either treatment alone, resulting in a 3-week longer survival time (Fig. 4*C*). These data provide support for use of E2F1/gemcitabine as a promising approach for treatment of pancreatic cancer. In addition, the correlation between *in vivo* and *in vitro* data underlines the biological and therapeutic relevance of the E2F1/p73 pathway in cancer therapy.
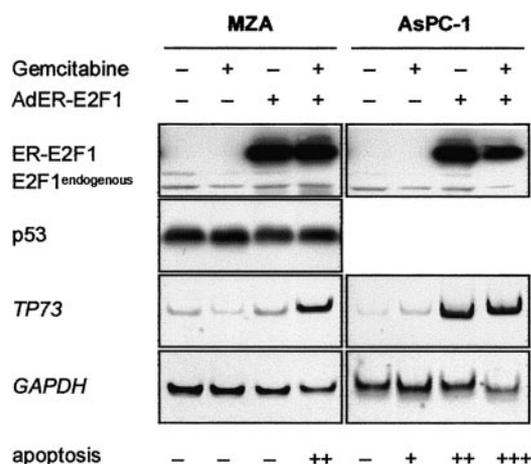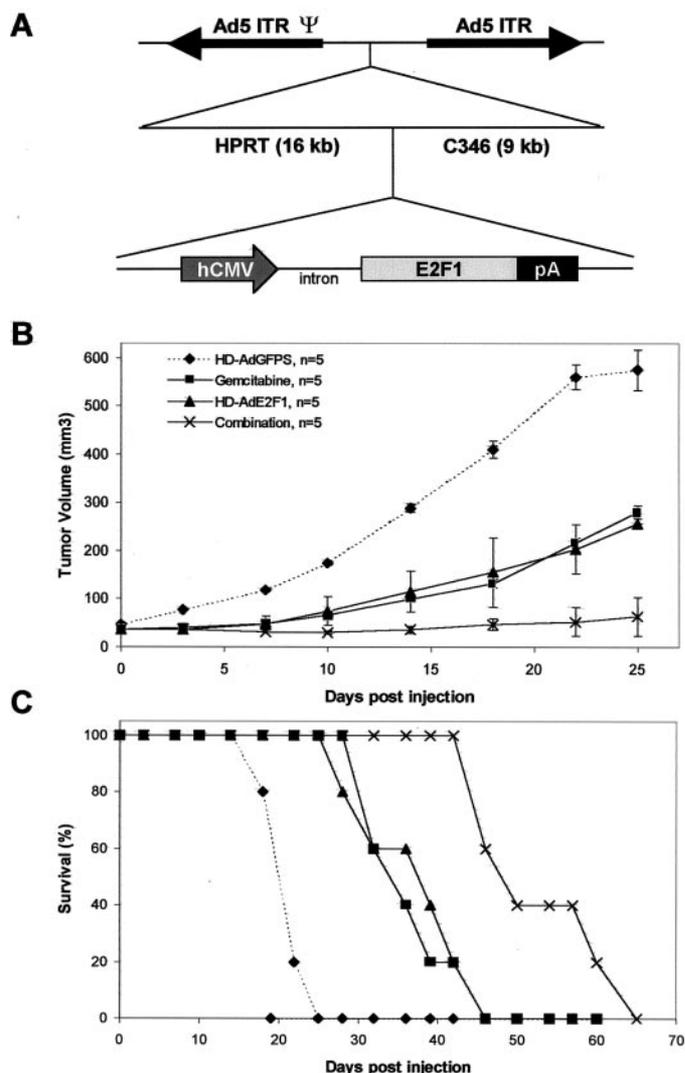
Acknowledgments

Fig. 3. Combination treatment of MZA and AsPC-1 cells correlates with *TP73* expression. Western blot of E2F1 and p53 and RT-PCR analysis of *TP73* expression 48 h after treatment with gemcitabine, AdER-E2F1, or the combination. Glyceraldehyde-3-phosphate dehydrogenase expression is shown as a control. The level of apoptosis induction is indicated as −, no apoptosis; +, >10%; ++, >20%; +++, >40% sub-G_1 cells.

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pCMVEGFP-Spectrin. We also thank Merck for providing 293Cre4 cells and AdLC8luc.

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