A Novel Role for Carcinoembryonic Antigen-Related Cell Adhesion Molecule 6 as a Determinant of Gemcitabine Chemoresistance in Pancreatic Adenocarcinoma Cells

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ABSTRACT

Most patients with pancreatic adenocarcinoma present with surgically incurable disease. Gemcitabine, the principal agent used to treat such patients, has little impact on outcome. Overexpression of carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6, a feature of this malignancy, is associated with resistance to anoikis and increased metastasis. The purpose of this study was to determine the role of CEACAM6 in cellular chemoresistance to gemcitabine. CEACAM6 was stably overexpressed in Capan2 cells, which inherently overexpress CEACAM6, by stable transfection of a CEACAM6 small interfering RNA-generating vector. The effects of modulating CEACAM6 expression on gemcitabine-induced cytotoxicity were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay, flow cytometric apoptosis quantification, caspase profiling, and Western analysis of cytoplasmic cytochrome c release. The roles of Akt and c-Src kinases as downstream targets of CEACAM6 signaling were examined. Stable overexpression of CEACAM6 in Capan2 increased gemcitabine chemoresistance, whereas CEACAM6 gene silencing in BxPC3 markedly increased the sensitivity of these cells to gemcitabine. Differential expression of CEACAM6 modulates Akt activity in a c-Src-dependent manner, and CEACAM6 overexpression appears to protect cells from cytotoxicity-induced caspase 3 activation and apoptosis.

INTRODUCTION

Highly aggressive locoregional invasion and early metastasis are characteristic of pancreatic adenocarcinoma, such that the majority of patients with this malignancy present with advanced, surgically resectable disease (1, 2). The nucleoside analog gemcitabine is one of the few agents shown to affect survival and quality of life in such patients. However, despite being the current standard of care for advanced pancreatic cancer, the clinical impact of gemcitabine remains modest (3, 4) due to a high degree of inherent and acquired chemoresistance.

Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6 is a glycosylphosphatidylinositol (GPI)-linked immunoglobulin superfamily member that is overexpressed in a variety of gastrointestinal malignancies (5, 6). Despite lacking an intracellular domain, CEACAM6 is a glycosylphosphatidylinositol (GPI)-linked immunoglobulin superfamily member that is overexpressed in a variety of gastrointestinal malignancies (5, 6). Despite lacking an intracellular domain, CEACAM6 is a feature of this malignancy, is associated with resistance to anoikis and increased metastasis. The purpose of this study was to determine the role of CEACAM6 in cellular chemoresistance to gemcitabine. CEACAM6 was stably overexpressed in Capan2 cells, which inherently overexpress CEACAM6, by stable transfection of a CEACAM6 small interfering RNA-generating vector. The effects of modulating CEACAM6 expression on gemcitabine-induced cytotoxicity were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay, flow cytometric apoptosis quantification, caspase profiling, and Western analysis of cytoplasmic cytochrome c release. The roles of Akt and c-Src kinases as downstream targets of CEACAM6 signaling were examined. Stable overexpression of CEACAM6 in Capan2 increased gemcitabine chemoresistance, whereas CEACAM6 gene silencing in BxPC3 markedly increased the sensitivity of these cells to gemcitabine. Differential expression of CEACAM6 modulates Akt activity in a c-Src-dependent manner, and CEACAM6 overexpression appears to protect cells from cytotoxicity-induced caspase 3 activation and apoptosis.

The purpose of this study was to characterize the contribution of CEACAM6 to pancreatic adenocarcinoma cellular chemoresistance to gemcitabine. Our results indicate that levels of CEACAM6 expression can modulate gemcitabine chemoresistance.

MATERIALS AND METHODS

Cells and Cell Culture. Capan2 and BxPC3 human pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and incubated in a humidified (37°C, 5% CO2) incubator, grown in 75-cm2 culture flasks, and passaged on reaching 80% confluence.

Expression Vector Construction and Transfection. Polyadenylated RNA was reverse transcribed using an anchored oligo(dT) primer with a Xhol restriction site. Double-stranded CEACAM6 cDNA was prepared and ligated into EcoRI adaptors, digested with Xhol, ligated into the pOTB7 vector, and transferred to the pRES-eGFP expression plasmid (termed pRES-CEACAM6). Cells were transfected with pRES-CEACAM6 or pRES-eGFP, which acted as a control, using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s protocol. Stable clones were selected by exposure to incrementally increasing concentrations of G418 (Gibco), isolated using cloning cylinders, and maintained in medium containing 0.8 mg/ml G418. Clones pRES-CEACAM6.1 and pRES-CEACAM6, which expressed the highest levels of CEACAM6, were studied. Constitutively active (myristoylated) Akt (myr-Akt) and dominant negative Src (Src [K296R/ Y528F]) expression constructs were obtained from Upstate (Waltham, MA). Transient transfection was performed using LipofectAMINE 2000.

Stable CEACAM6 RNA Interference. CEACAM6-specific and single base mismatch small interfering RNA expression constructs were generated by ligation of inserts (containing Xhol and XbaI overhangs sites) targeting the following sequences into the pSuppressorNeo expression vector in accordance with the manufacturer’s instructions (Imagenex, San Diego, CA): CEACAM6 (psCEACAM6), 5′-CCGGACAGTCCATTGATA-3′, and control (psControl), 5′-CCGGACAGTCCATTGATA-3′. Correct insert ligation was confirmed by absence of linearization by Sall. Cells were transfected as described. Stable transfected clones were established using G418. Clones stably transfected with empty vector, psi (−), and psControl served as controls.

Cytotoxicity Assay. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Treveniq Inc., Gaitersburg, MD) in accordance with the manufacturer’s instructions. Results of the MTT assay have been shown to correlate well with [3H]thymidine incorporation in pancreatic cancer cell lines (12). Logarithmically growing cells were plated at 5 × 104 cells/well in 96-well plates, allowed to adhere over night, and cultured in the presence or absence of 0–10 μM gemcitabine. Gemcitabine-induced cytotoxicity was determined after 48 h of exposure. Plates were read using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm corrected to 650 nm and normalized to controls. Each independent experiment was performed 3 times, with 10 determinations for each condition tested. The concentration of gemcitabine required to inhibit proliferation by 50% (IC50) was calculated from these results. At identical time points, cells were trypsinized to form a single cell suspension. Intact cells, determined by trypan blue exclusion, were counted using a Neubauer hemocytometer (Hauser Scientific, Horsham, PA). Cell counts were used to confirm MTT results.

Apoptosis Staining. After gemcitabine treatment, cells were washed and resuspended in 0.5 ml of PBS, and 1 μl/ml YO-PRO-1 and propidium iodide were added (Vybrant Apoptosis Assay Kit #4; Molecular Probes, Eugene, OR). Cells were incubated for 30 min on ice and then analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ), measuring fluorescence...
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Fig. 1. A. Western blot analysis of parental Capan2 cells, pRES-eGFP, pRES-CEACAM6.1, and pRES-CEACAM6.2 transfectants confirmed stable overexpression of CEACAM6 in both pRES-CEACAM6.1 and pRES-CEACAM6.2. Representative blot (top panel) and mean ± SD densitometric values (normalized to actin, bottom panel) from three independent experiments are shown. *, P < 0.05 versus Capan2 and pRES-eGFP. B. Effect of CEACAM6 overexpression on gemcitabine chemosensitivity, quantified by eGFP. B. Mean values from triplicate experiments with 10 determinations/condition are shown. *, P < 0.05 versus Capan2 and pRES-eGFP. C. Increased gemcitabine chemoresistance was associated with a lower apoptotic fraction in pRES-CEACAM6.1 and pRES-CEACAM6.2 transfectants after exposure to 1 μM gemcitabine for 48 h. Values are means ± SD from triplicate experiments. *, P < 0.05 versus Capan2 and pRES-eGFP.
Charles River Laboratories (Wilmington, MA). Mice were housed in microisolation cages with autoclaved bedding in a specific pathogen-free facility with 12-h light/dark cycles. Animals received water and food ad libitum and were observed for signs of tumor growth, activity, feeding, and pain in accordance with the guidelines of the Harvard Medical Area Standing Committee on Animals. Mice were anesthetized with i.p. ketamine (200 mg/kg) and xylazine (10 mg/kg) and inoculated with $10^6$ pancreatic adenocarcinoma cells in 75 $\mu$l of PBS by s.c. implantation. Treatment commenced 20 days after implantation, when tumors were approximately 50 mm$^3$ in volume. Mice received gemcitabine (150 mg/kg) in 100 $\mu$l of PBS vehicle by twice-weekly i.p. injection. Tumor dimensions were measured weekly using micrometer calipers. Tumor volumes were calculated using the following formula: $Volume = \frac{1}{2} a \times b^2$, where $a$ and $b$ represent the larger and smaller tumor diameters, respectively. After 6 weeks of treatment and 4 days after the final gemcitabine injection, necropsy was performed, and primary tumors were excised, formalin fixed, and paraffin embedded, and stained by terminal deoxynucleotidyl transferase-mediated nick end labeling.

**Statistical Analysis.** Differences between groups were analyzed using Student’s $t$ test, multifactorial ANOVA of initial measurements, and Mann-Whitney $U$ test for nonparametric data, as appropriate, using Statistica 5.5 software (StatSoft, Inc., Tulsa, OK). In cases in which averages were normalized to controls, the SDs of each nomenclator and denominator were taken into account in calculating the final SD. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Effect of CEACAM6 Overexpression on Gemcitabine Chemosensitivity.** We have reported previously that pancreatic adenocarcinoma cells differentially express CEACAM6 (11). BxPC3 cells markedly overexpress CEACAM6, relative to Capan2 cells (11, 15), and exhibit greater chemoresistance to gemcitabine (16). We directly determined the effect of CEACAM6 overexpression on gemcitabine chemoresistance by establishing clones of Capan2 cells that stably overexpressed CEACAM6, after transfection with the pIRES-CEACAM6 expression vector. Capan2 cells stably transfected with pIRES-eGFP served as controls. The gemcitabine IC$_{50}$ was determined for each clone by MTT cytotoxicity assay. Stable overexpression of CEACAM6 by the clones pIRES-CEACAM6.1 and pIRES-CEACAM6.2 was confirmed by Western blot analysis (Fig. 1A). Both pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants demonstrated increased gemcitabine chemoresistance, compared with parental Capan2 cells and pIRES-eGFP transfectants (Fig. 1B). Exposure to $1\mu$m gemcitabine for 48 h resulted in a lower apoptotic fraction in pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants, relative to control cells (Fig. 1C).

The effect of CEACAM6 overexpression on cellular proliferation in the absence of gemcitabine was determined and, consistent with our previously reported observation that CEACAM6 gene silencing does not affect cellular proliferation (11), we observed no difference in the doubling time of pIRES-CEACAM6.1, pIRES-CEACAM6.2, and pIRES-eGFP transfectants and Capan2 cells.

**Effect of CEACAM6 Gene Silencing on Gemcitabine Chemosensitivity.** Next, we sought to determine whether suppression of CEACAM6 expression would alter the sensitivity of BxPC3 pancreatic adenocarcinoma cells to gemcitabine. To induce stable suppression of CEACAM6 expression, BxPC3 cells were stably transfected...
with the CEACAM6-specific small interfering RNA expression vector (psiCEACAM6) and selected on the basis of their resistance to G418. Cells stably transfected with the empty vector [psi(−)] and a vector encoding a single base mismatch small interfering RNA (psiControl) served as controls. Stable suppression of CEACAM6 expression (up to 90%) was confirmed by Western blot analysis (Fig. 2A). Consistent with our previous observations, stable suppression of CEACAM6 did not affect cellular proliferation in the absence of gemcitabine. However, psiCEACAM6 transfectants exhibited a significant decrease in their chemoresistance to gemcitabine, compared with both psi(−) and psiControl transfectants (Fig. 2B). Relative to control cells, psiCEACAM6 transfectants exposed to 1 μM gemcitabine for 48 h exhibited a higher apoptotic fraction (Fig. 2C).

Modulation of CEACAM6 Expression Affects Gemcitabine-Induced Caspase 3 Activation, but Not Cytochrome c Release. Mitochondrial damage with release of cytochrome c is implicated in cell death signaling pathways and occurs in pancreatic adenocarcinoma cells after exposure to gemcitabine (17). Gemcitabine-induced cytochrome c release is associated with caspase 3 activation in human adenocarcinoma cells (18, 19). The apoptosome, a multiprotein complex comprising Apaf-1, cytochrome c, and initiator caspase 9, functions to activate executioner caspase 3 downstream of mitochondria in response to apoptotic signals (20, 21). Gemcitabine-induced activation of caspase 3 was significantly reduced in pIRES-CEACAM6.1 and pIRES-eGFP transfectants, relative to pIRES-eGFP and parental Capan2 cells. Similarly, suppression of CEACAM6 expression in BxPC3 pIRES-CEACAM6 transfectants was associated with increased gemcitabine-induced caspase 3 activation, compared with BxPC3 cells and psi(−) and psiControl transfectants. (Fig. 3A). The psiControl vector had no effect on gemcitabine-induced caspase 3 activity. Interestingly, there was no significant difference in the degree of gemcitabine-induced cytochrome c release in pIRES-CEACAM6.1, pIRES-CEACAM6.2, or pIRES-eGFP transfectants (Fig. 3B). Similarly, there was no significant difference in gemcitabine-induced cytochrome c release among BxPC3 cells and psi(−), psiControl, and psiCEACAM6 transfectants (Fig. 3C).

In view of these findings, we performed cell-free in vitro assessment of the effect of CEACAM6 overexpression on cytochrome c-induced activation of caspase 3. Addition of cytochrome c and dATP to cytosolic extracts results in caspase 3 activation (22). Cell extracts prepared from pIRES-CEACAM6.1 and pIRES-CEACAM6.2 transfectants demonstrated significantly lower levels of caspase 3 activity after addition of cytochrome c and dATP than those from pIRES-eGFP transfectants or Capan2 cells. Conversely, BxPC3 psiCEACAM6 transfectants exhibited higher levels of caspase 3 activity after addition of cytochrome c and dATP (Fig. 4). We confirmed the importance of caspase activation in mediating the enhanced gemcitabine-induced apoptosis observed in psiCEACAM6 by exposing cells to the caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk; 100 μM), which reduced the mean apoptotic fraction of psiCEACAM6 cells exposed to gemcitabine from 28% to 9% (P < 0.05). This finding, taken together with the previous observations, suggests that CEACAM6 overexpression may exert a cytoprotective effect, at least in part, by inducing inhibition of caspase 3 activation in response to cytochrome c release, an effect that appears to occur downstream of cytochrome c release.
Effect of CEACAM6 Overexpression on Akt Kinase Activity.

Akt represents an important determinant of gemcitabine chemoresistance in pancreatic adenocarcinoma (23) and is a central regulator of cell proliferation in pancreatic adenocarcinoma cells, including BxPC3 (24). Akt is also of functional importance in the regulation of caspase activity (13, 25). Previously, we have reported that CEACAM6 gene silencing suppresses Akt Ser-473 phosphorylation, a marker of Akt activation (11). We therefore assessed the effect of CEACAM6 overexpression on Akt kinase activity because this may account for the increased chemoresistance of cells overexpressing CEACAM6. In addition, we assessed the effect of CEACAM6 gene silencing on Akt activity in BxPC3 cells. Akt activity was quantified using an in vitro kinase assay that detects phosphorylation of GSK-3, a physiological substrate of Akt. BxPC3 cells demonstrate greater in vitro Akt activity than Capan2 cells. Furthermore, overexpression of CEACAM6 significantly increased Akt activity in Capan2 cells. Conversely, psiCEACAM6 BxPC3 transfectants demonstrated a lower level of Akt activity than psiControl BxPC3 transfectants (Fig. 5A).

To determine whether Akt was able to compensate for the effect of suppressing CEACAM6 expression on gemcitabine chemoresistance, we introduced a constitutively active (myristoylated) p-USE-amp-based Akt expression construct (myr-Akt) into psiCEACAM6 BxPC3 transfectants. Cells transfected with the empty vector [p-USE-amp(−)] served as controls. Transfection of myr-Akt increased Akt activity and returned the gemcitabine IC50 to values close to those of psiControl transfectants (Fig. 5, B and C). Together, these observations indicate that levels of CEACAM6 modulate Akt and that this contributes to the chemoprotective effect of CEACAM6 overexpression.

CEACAM6-Dependent Akt Activation Is Mediated by c-Src.

It has recently been recognized that c-Src directly activates Akt through an interaction between its SH3 domain and a conserved proline-rich motif in the COOH-terminal regulatory region of Akt (26). Furthermore, Src family kinases have been reported to associate with CEACAM family members (27). Increased c-Src activity, detected by

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in vitro kinase assay, was observed in lysates from both pRES-CEACAM6.1 and pRES-CEACAM6.2 transfectants, compared with control cells (Fig. 6A). We therefore assessed the effect of transfection of dominant negative c-Src on Akt activity after CEACAM6 overexpression. Dominant negative c-Src almost completely abolished the increase in Akt activity in both pRES-CEACAM6.1 and pRES-CEACAM6.2 (Fig. 6B), suggesting that CEACAM6 overexpression increases Akt activity in a c-Src-dependent manner.

**In Vivo Chemoresistance.** Mice implanted with either BxPC3 psiCEACAM6 or psiControl transfectants were treated with gemcitabine over a 6-week period. Tumor volume was measured weekly. Mice implanted with psiCEACAM6 transfectant xenografts exhibited slower tumor growth than those implanted with psiControl transfectants (Fig. 7A). Terminal deoxynucleotidyl transferase-mediated nick end labeling staining of the tumor specimens revealed a 1.8-fold higher level of apoptosis in psiCEACAM6 transfectant tumors than in psiControl transfectant tumors (Fig. 7B).

**DISCUSSION**

Pancreatic adenocarcinoma is among the most chemoresistant of malignancies, and there exists an urgent need to establish new therapeutic targets and approaches. In this study, we have identified the GPI-anchored protein CEACAM6 as a determinant of pancreatic adenocarcinoma cellular chemoresistance to gemcitabine. Despite lacking transmembrane and intracellular domains, CEACAM6 is able to influence intracellular events critical to the regulation of apoptosis. Our study is the first to demonstrate a role for CEACAM6 in gemcitabine chemoresistance and provides evidence that Akt may convey the chemoprotective effect of CEACAM6 overexpression in a c-Src-dependent manner.

Growing evidence implicates CEACAM6 in gastrointestinal cancer progression (6, 8, 9, 11). We have shown previously that modulation of CEACAM6 expression influences cellular susceptibility to the proapoptotic stimulus of anchorage-independent culture (11). In the current study, overexpression of CEACAM6 resulted in increased gemcitabine chemoresistance, and this, together with our observation that gemcitabine-induced apoptosis is attenuated by knockdown of CEACAM6 expression, is consistent with a model in which CEACAM6 determines cellular susceptibility to apoptosis. CEACAM6 is not alone among GPI-anchored proteins in having importance in the regulation of apoptosis. Bounhar et al. (28) reported that the GPI-linked prion protein potently inhibits Bax-induced apoptotic cell death in human primary neurons. GPI-defective Jurkat T cells are reportedly more susceptible to Fas-mediated apoptosis (29), and antibody-mediated targeting of the GPI-linked CAMPATH-1H (CD52) antigen has been shown to result in apoptosis of human lymphoma cells (30). Although it appears increasingly likely that GPI-linked proteins play a part in regulating apoptosis, the mechanisms through which this family of proteins influences intracellular apoptotic pathways have received relatively little attention.

Considerable evidence derived from work performed in a variety of malignancies, including pancreatic adenocarcinoma, indicates that Akt plays an important role in the regulation of cellular apoptosis (13, 23, 31–33). Active Akt appears to protect cells from a variety of apoptotic stimuli, including exposure to gemcitabine (13, 23, 32, 33). The caspase cascade of proteolytic enzymes comprises initiator and executioner elements, which, after activation, lead to degradation of intracellular targets resulting in apoptotic cell death. Active Akt has been shown to phosphorylate initiator caspase 9 directly, preventing its activation (25). Inhibition of this initiator caspase may interfere with apoptosome function and impair activation of effector caspase 3. Akt has also been reported to inhibit caspases by posttranslational modification of an as yet unidentified cytosolic factor located downstream of cytochrome c release and upstream of caspase 9 activation (13). The results of our study are consistent with this paradigm of Akt-mediated caspase regulation. Firstly, we have shown that Akt activity is increased by CEACAM6 overexpression and decreased by CEACAM6 gene silencing. Secondly, the effects of modulating CEACAM6 expression appear to influence the apoptotic pathway downstream of cytochrome c release because altering CEACAM6 expression influences cytochrome c-induced caspase activity but does not affect cytoplasmic cytochrome c release. The mechanisms underlying these observations will require further investigation, but Akt would appear to be a candidate mediator of CEACAM6-dependent apoptotic regulation.

The tyrosine kinase c-Src has recently been shown to induce activating phosphorylation of Akt through a direct interaction with the c-Src SH3 domain and a conserved proline-rich motif in the COOH-terminal regulatory region of Akt (26). The previously reported association of Src family kinases with CEACAM6 is consistent with the increased c-Src activity we detect in lysates from both pRES-CEACAM6.1 and pRES-CEACAM6.2 transfectants. The ability of a dominant negative Src expression construct to impair the increased Akt activity seen in CEACAM6-overexpressing clones further supports the role of c-Src in mediating CEACAM6-dependent Akt activation. In a recent study, we illustrated the functional importance of c-Src-Akt signaling in gemcitabine chemoresistance. Treatment of pancreatic adenocarcinoma cells with the Src family kinase inhibitor 4-amin-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and transfection of a dominant negative Src expression construct were each sufficient to impair gemcitabine chemoresistance. Conversely, overexpression of active Src was chemoprotective in pancreatic adenocarcinoma cells. Src inhibition was also associated decreased Akt (Ser-473) phosphorylation status, consistent with results of the present study (34).

Although RNA interference induced by transfection of small interfering RNA oligonucleotide is emerging as a powerful tool, this approach is limited by its transient nature (35). By stably transfecting cells with a short hairpin expression vector, as was used in this study, this limitation can be overcome (36). The ability to sustain a cell line that stably underexpresses CEACAM6 demonstrates that this molecule is not essential for cell survival under standard culture conditions and is consistent with our previously reported observations that suppression of CEACAM6 expression does not affect cellular proliferation or apoptosis in monolayer culture (11).

In summary, our findings indicate that overexpression of CEACAM6 can protect pancreatic adenocarcinoma cells from gemcitabine-induced cytotoxicity. Conversely, knockdown of CEACAM6 expression has a chemosensitizing effect. In addition to functioning as an intercellular adhesion molecule, CEACAM6 may constitute part of an antiapoptotic survival pathway in these cells. This cytoprotective pathway may contribute to gemcitabine chemoresistance, and targeting this pathway may be a potential therapeutic strategy in this highly treatment-resistant disease.

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