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Treatment of Terminal Peritoneal Carcinomatosis by a Transducible p53-Activating Peptide

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Advanced-stage peritoneal carcinomatosis is resistant to current chemotherapy treatment and, in the case of metastatic ovarian cancer, results in a devastating 15%–20% survival rate. Therapeutics that restore genes inactivated during oncogenesis are predicted to be more potent and specific than current therapies. Experiments with viral vectors have demonstrated the theoretical utility of expressing the p53 tumor suppressor gene in cancer cells. However, clinically useful alternative approaches for introducing p53 activity into cancer cells are clearly needed. It has been hypothesized that direct reactivation of endogenous p53 protein in cancer cells will be therapeutically beneficial, but few tests of this hypothesis have been carried out in vivo. We report that a transducible D-isomer RI-TATp53C peptide activates the p53 protein in cancer cells, but not normal cells. RI-TATp53C peptide treatment of preclinical terminal peritoneal carcinomatosis and peritoneal lymphoma models results in significant increases in lifespan (greater than 6-fold) and the generation of disease-free animals. These proof-of-concept observations show that specific activation of endogenous p53 activity by a macromolecular agent is therapeutically effective in preclinical models of terminal human malignancy. Our results suggest that TAT-mediated transduction may be a useful strategy for the therapeutic delivery of large tumor suppressor molecules to malignant cells in vivo.

Introduction

Most patients who succumb to cancer do so not from primary tumor burden, but from metastatic disease (Fidler 2003). For example, advanced-stage peritoneal carcinomatosis (e.g., from metastatic ovarian and breast cancer) and disseminated peritoneal lymphomas are often resistant to current chemotherapy treatment (Parsons et al. 1996). Posttreatment survival rates for patients presenting with metastatic ovarian peritoneal carcinomatosis or lymphoma are less than 20% and less than 50%, respectively (Lam and Zhao 1997; Deppe and Baumann 2000; Hofstra et al. 2000). Consequently, the development of novel therapeutic strategies to reverse these numbers is clearly warranted.

A significant effort has been aimed at understanding the function of tumor suppressor gene pathways that are genetically and epigenetically altered during oncogenesis (Macleod 2000). One rationale for the study of tumor suppressor pathways is the hypothesis that reconstitution of these pathways in cancer patients will be therapeutically beneficial (Macleod 2000). The p53 tumor suppressor protein induces growth arrest and apoptosis in response to cellular stress ( Vousden and Lu 2002). Mutation of genes in the p53 pathway is thought to be nearly universal in human cancer ( Vousden and Lu 2002). Thus, any strategy designed to restore p53 activity in tumor cells will likely be an effective means of inducing cancer cell death and will be applicable to a large fraction of cancer patients. The inability of large tumor suppressor proteins, all of which are intracellular, to cross the plasma membrane precludes the therapeutic administration of recombinant tumor suppressors in a manner analogous to administration of extracellular biological therapeutics (e.g., insulin or G-CSF). Thus, the development of an efficient methodology for restoring tumor suppressor function to cancer cells in vivo remains a challenge for both basic and clinical researchers.

Gene therapy approaches aimed at restoring tumor suppressor function have been extensively investigated. Both viral and nonviral vectors have been employed to express exogenous tumor suppressor genes, such as p53, in cancer cells (McCormick 2001). Although gene therapy may be useful under certain conditions, problems associated with immunogenicity and lack of systemic biodistribution to disseminated metastases are likely to curtail its anticancer efficacy (McCormick 2001).

Delivery of macromolecules by protein transduction has recently emerged as an alternative methodology for directly introducing tumor suppressor proteins into cancer cells in vivo. Several small cationic peptides, including TAT, Antp, and polyArg (referred to as protein transduction domains [PTDs]), are capable of traversing the plasma membrane and entering the cytoplasm of cells by a concentration-dependent, but receptor-independent, macropinocytic mechanism.
Activation of p53 by Transducible Retro-Inverso D-Isomer Peptide

Results

Activation of p53 by Transducible Retro-Inverso D-Isomer p53C’ Peptide

Although PTDs solve one major obstacle to the use of intracellular peptides as therapeutics, the susceptibility of peptides to degradation in vivo remains problematic. To circumvent the problem of proteolytic degradation, we synthesized a retro-inverso version of the parental p53C’ peptide by inverting the peptide sequence, using D-isomer residues, and adding the TAT PTD to obtain a transducible RI-TATp53C’ peptide (Figure 1A). This double inversion of peptide structure often leaves the surface topology of the sidechains intact and has been used extensively to stabilize biologically active peptides for in vivo applications (Chorev and Goodman 1993). Because of their greater stability, retro-inverso peptides often display increased potency.

To determine whether the RI-TATp53C’ peptide retained functionality, we compared the transducible parental L-isomer p53C’TAT and D-isomer RI-TATp53C’ peptides for the ability to induce a cell cycle arrest (Figure 1B). Treatment of murine TA9/St mammary carcinoma cells (which express wild-type p53) with either the L-isomer p53C’TAT or D-isomer RI-TATp53C’ peptides resulted in a concentration-dependent G1 cell cycle arrest (Figure 1B and 1C). The control D-isomer mutant peptide had little to no effect (Figure 1B and 1C). Compared to the L-isomer p53C’TAT, the D-isomer RI-TATp53C’ peptide induced a stronger cell cycle arrest at substantially lower concentrations (Figure 1C). A single administration of the L-isomer p53C’TAT peptide partially arrested cells for 24 h, but by 48 h cells had reentered the cell cycle (Figure 1C). In contrast, a single dose of the D-isomer RI-TATp53C’ peptide was sufficient to sustain a G1 arrest for greater than 7 d (Figure 1C; data not shown).

To ascertain whether sustained arrest required the continuous presence of RI-TATp53C’ peptide, TA9/St cells were treated with peptide or vehicle for 2 d and then replated under mitogenic conditions in the absence of peptide. Peptide-treated tumor cells formed less than 1% as many colonies as vehicle-treated cells (Figure 1D). This observation suggested that RI-TATp53C’ peptide induced a permanent growth arrest in TA9/St cells. We therefore assayed RI-TATp53C’ peptide-treated cells for induction of senescence, a state of terminal arrest that can be induced by p53 activation (Roninson et al. 2002). By 6 d after peptide addition, greater than 80% of viable TA9/St cells were positive for acidic β-galactosidase activity (Figure 1E), the standard marker of senescence (Roninson et al. 2002). The treated cells also displayed other features of senescence (Roninson et al. 2002), including increased size, increased granularity, and a flattened morphology (Figure 1E; data not shown). These observations suggest that treatment of mammary carcinoma cells with the RI-TATp53C’ peptide induces hallmarks of p53 activation, namely a G1 cell cycle arrest followed by induction of senescence.

We next investigated the ability of the RI-TATp53C’ peptide to transcriptionally activate p53-responsive genes. We transiently transfected p53 null human H1299 lung carcinoma cells with p53-dependent luciferase reporter plasmid (PG13-Luc) and either wild-type p53 expression plasmid or empty vector. The use of p53 null cells allows for negative controls that are not possible in cells expressing endogenous p53. As expected, we observed a p53-dependent induction of luciferase activity in cells transfected with p53 expression vector (Figure 2A). However, RI-TATp53C’ peptide treatment of cells transfected with p53 expression vector resulted in a significant increase in p53-dependent luciferase activity (Figure 2A, left). Consistent with observations in TA3/St cells (see Figure 1B and 1C), mutant peptide and L-isomer p53C’TAT displayed substantially reduced potency in this assay when compared to RI-TATp53C’ peptide (data not shown). Importantly, RI-TATp53C’ peptide treatment of cells transfected with empty vector and luciferase plasmids caused no increase in p53 target promoter activity (Figure 2A). In addition, RI-TATp53C’ peptide activated p53-dependent transcription in SW480 colon carcinoma cells expressing a p53 DNA contact mutant (R273H) and in H1299 p53 null colon carcinoma cells transfected with a p53 DNA contact mutant (R248Q and R273H) (Figure 2A, right), though to a lesser extent than in the presence of wild-type p53 (Figure 2A, left). These observations show that RI-TATp53C’ peptide
retains the ability to specifically activate p53-dependent gene transcription.

To confirm that the RI-TATp53C’ peptide inhibited tumor cell proliferation in a p53-dependent fashion, we compared parental p53+/+ HCT116 colorectal carcinoma cells to HCT116 cells that were rendered p53-deficient at both loci by targeted genetic recombination (Bunz et al. 1998). Treatment of wild-type p53+/+HCT116 cells with RI-TATp53C’ peptide inhibited cell proliferation in a dose-dependent manner (Figure 2B). In contrast, RI-TATp53C’ peptide treatment of p53-deficient HCT116 cells did not significantly alter the number of viable cells. p53-deficient human H1299 lung adenocarcinoma cells also failed to respond to the RI-TATp53C’ peptide or vehicle for 2 d, replated, and allowed to proliferate in the presence of serum for 10 d. Colonies were then stained with methylene blue.

(D) Induction of a senescence-like phenotype in TA3/St cells by RI-TATp53C’. Cells were treated with RI-TATp53C’ peptide and stained for acidic β-galactosidase activity.

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Systemic Delivery of RI-TATp53C’ Peptide Inhibits Solid Tumor Growth

We (Schwarze et al. 1999) and others (Datta et al. 2001; Harada et al. 2002) have previously shown that intraperitoneal (IP) administration of TAT–fusion peptides and proteins results in systemic delivery in animal models. Consistent with
these observations, IP injection of a biotinylated RI-TATp53C’ peptide into mice harboring subcutaneous tumors resulted in distribution of the peptide throughout the tumor (Figure 3A).

We next tested the ability of IP administration of RI-TATp53C’ peptide to inhibit the growth of distant solid tumors in immune competent mice. Subcutaneous tumors in mice receiving either vehicle or mutant peptide grew rapidly, reaching an average volume of nearly 600 mm³ by the end of treatment (Figure 3B). In contrast, tumors in mice treated with wild-type RI-TATp53C’ peptide were significantly retarded in growth and reached a final mean volume less than 50% that of tumors in the control-treated mice ($p < 0.01$) (Figure 3B). These observations demonstrate that systemic delivery of RI-TATp53C’ peptide in immune competent mice can significantly inhibit the growth of an aggressively proliferating solid tumor at a distant site.

**RI-TATp53C’ Peptide Treatment of Terminal Peritoneal Carcinomatosis**

Because of their encapsulation and ectopic site of growth, subcutaneous tumors fail to replicate many of the features of terminal human cancer. We therefore tested the efficacy of RI-TATp53C’ peptide in a terminal peritoneal carcinomatosis mouse model that more closely resembles metastatic human disease. TA3/St carcinoma cells inoculated into the peritoneum of immune competent, syngeneic A/Jax (A/J) mice proliferated in a rapid logarithmic fashion, doubling in 24 h and increasing their numbers 100-fold 5 d postinoculation (Nagy et al. 1993). This aggressive, terminal peritoneal carcinomatosis model of human disease has been used extensively to study the pathophysiology of peritoneal tumor growth (Nagy et al. 1993, 1995).

We assayed the ability of the RI-TATp53C’ peptide to alter the tumor burden and increase the longevity of mice harboring TA3/St peritoneal carcinomatosis. Vehicle-treated mice rapidly succumbed to peritoneal tumor burden with a mean survival time of 11 d (Figure 4A). Mice treated with control mutant peptide succumbed to their tumor burden with similar kinetics and a mean survival time of 10 d (Figure 4A). In contrast, peritoneal tumor-bearing mice treated with wild-type RI-TATp53C’ peptide lived on average more than 70 d after tumor inoculation (Figure 4A), a greater than 6-fold increase in lifespan over mutant peptide- or vehicle-treated mice ($p < 10^{-6}$). These observations demonstrate the ability of transducible peptides to significantly extend survival in a mouse model of terminal peritoneal carcinomatosis.

We next investigated the biological consequences of peptide treatment to tumor cells in vivo. Peritoneal-TA3/St tumor-bearing mice were given daily injections of wild-type RI-TATp53C’ peptide or vehicle control. Mice were sacrificed 3 d after tumor cell inoculation for assessment of tumor...
burden. Vehicle-treated mice contained a significant tumor burden of recoverable dividing TA3/St tumor cells (Figure 4B). In contrast, mice treated with wild-type RI-TATp53C peptide showed a dramatic reduction in tumor cell number, suggesting that RI-TATp53C peptide treatment extended survival by directly inhibiting overall tumor proliferation. Consistent with cell culture studies, cell cycle analysis of tumor cells from peptide-treated mice showed an increase in G1 phase of the cell cycle (data not shown).

RI-TATp53C Peptide Treatment of Terminal Peritoneal Lymphoma

To broaden these results, we also tested the efficacy of the RI-TATp53C peptide in a mouse model of aggressive, disseminated peritoneal lymphoma. Wild-type RI-TATp53C peptide, but not mutant peptide, induced G1 phase accumulation and substantial apoptosis in Namalwa human lymphoma cells (Figure 5A). When injected IP into SCID (severe combined immune deficiency) mice, Namalwa cells proliferate in the peritoneum and disseminate to other locations (e.g., spleen, lymph nodes, and blood [de Menezes et al. 1998]), modeling human B-cell lymphoma (Bertolini et al. 2000). Mice harboring peritoneal lymphoma succumbed to tumor burden with similar kinetics when treated with either vehicle or mutant peptide, with a mean survival time of 35 d and 33 d, respectively (Figure 5B). In contrast, wild-type RI-TATp53C peptide treatment resulted in 50% long-term survival (p < 0.0007) (Figure 5B), with six of 12 treated mice still healthy at more than 200 d after tumor cell injection. Taken together, these observations demonstrate that in models of terminal metastatic human disease, transducible p53-activating peptides can modulate tumor biology in vivo, resulting in significantly decreased tumor burden, increased lifespan, and long-term disease-free survival.

The subset of RI-TATp53C peptide-treated animals that succumbed to peritoneal carcinomatosis could have failed treatment either because of the emergence of peptide-resistant tumor cells or because of insufficient treatment
duration. To distinguish between these two possibilities, we isolated TA3/St and Namalwa cells from animals that failed treatment. In both cases, the cells readily proliferated in culture under the same conditions as the parental cell population (data not shown). RI-TATp53C peptide treatment of reconstituted TA3/St cells induced a G1 arrest similar in extent to that of the parental cell line (Figure 6A). RI-TATp53C peptide treatment of reconstituted Namalwa cells also inhibited the viability of both parental and reconstituted cells to the same degree (Figure 6B). These observations demonstrate that treatment failure is not due to acquisition of RI-TATp53C peptide resistance and suggests that an extended treatment protocol (greater than 12 d) may lead to a further enhancement of survival in these preclinical cancer models.

**Discussion**

Advanced-stage peritoneal carcinomatosis and disseminated peritoneal lymphomas are often resistant to current chemotherapy treatment (Parsons et al. 1996), and new strategies for treating these diseases are clearly needed. The need to develop different therapeutic modalities to restore tumor suppressor function is acutely illustrated by the current limitations of viral/DNA-based strategies for delivering tumor suppressor genes to cancer cells in patients (McCormick 2001). Here we show that macromolecular biological cargo can be delivered via TAT-mediated transduction in order to modulate tumor biology in vivo. Specifically, we find that delivery of a transducible p53-activating peptide in sensitive tumor cells inhibits solid tumor growth in vivo (see Figure 3) and dramatically extends survival (greater than 6-fold), yielding disease-free animals in terminal peritoneal cancer models of human metastatic disease (see Figures 4 and 5).

The vast majority of tumors express either wild-type p53...
of p53 remain (Fidler et al., 2002), demonstrates that transducible agents can effectively treat rigorous models of terminal cancer.

Current clinical use of macromolecular biological therapeutics is limited to agents that have an extracellular mode of action. The preclinical data presented here demonstrate a proof-of-concept that intracellular delivery of biologically active macromolecular cargo by TAT-mediated transduction can modify specific pathways in vivo and that this approach potentially serves as a foundation for the generation of new classes of intracellular biological therapeutics.

Materials and Methods

Cell culture and flow cytometry. TA3/St (gift of W. G. Kaelin), H1299 (gift of R. K. Brachmann), and human foreskin fibroblast (M. Haas) cells were maintained in DMEM plus 10% fetal bovine serum (FBS) and penicillin-streptomycin (P/S). Namalwa cells (American Type Culture Collection, Manassas, Virginia, United States) were maintained in RPMI plus 10% FBS, P/S, HCT116 cells (gift of B. Vogelstein) were grown in McCoy's medium plus 10% FBS, P/S. All cells were maintained at 37°C in 5% CO₂. Short-term cell viability was assessed by counting Trypan blue-excluding cells on a hemocytometer. Long-term cell viability was assessed by colony formation assay. After serial dilution and 10 d of culture, colonies were washed in PBS and stained with 1% methylene blue. Cellular senescence was assessed by Gal-6 staining as previously described (Schwarz et al., 1999), except for the use of PBS (pH 6.0). For cell cycle analysis, TA3/St cells were treated with 0.25–10 μM peptide and Namalwa cells with 40 μM peptide. DNA was stained 24 h later with 10 μg/ml propidium iodide in 0.5% NP-40 (TA3/St cells) or Draq5 (Namalwa cells) per the manufacturer's instructions (Qiogene, Carlsbad, California, United States). DNA profiles were analyzed using a FACScan and CellQuest software (Becton Dickinson, Palo Alto, California, United States).

Peptide synthesis. Peptides were synthesized by standard Fmoc chemistry on an ABI 435A Peptide Synthesizer (Applied Biosystems, Foster City, California, United States). Crude peptides were purified by reverse-phase HPLC over a C18 preparatory column (Varian, Palo Alto, California, United States). The identity of all peptides was confirmed by mass spectrometry.

Promoter activity assays. In a 96-well dish, 4 × 10⁴ cells were plated per well. The next day, H1299 cells were transfected with 1.5 ng of TK-Renilla (Promega, Madison, Wisconsin, United States), 200 ng of PG13-Luc, and one of the following: 0.3 ng of empty vector, 0.3 ng of p53 expression vector, or 1 ng mutant of p53 expression vector (gift of R. K Brachmann). SW480 cells were transfected with 25 ng of TK-Renilla and 250 ng of PG13-Luc reporter plasmid. Cells were all transfected using Lipofectamine 2000 per the manufacturer's protocol (Invitrogen, Carlsbad, California, United States). After 5 h, the transfection medium was removed and peptides were added to cells. Luciferase activity was measured 24 h later with the Dual Luciferase Reporter Assay System per the manufacturer's instructions (Promega).

Animal tumor models. For TA3/St tumor models, 4- to 8-wk-old immune competent C57BL/6 female mice were obtained from Jackson Laboratory (Bar Harbor, Maine, United States). Solid TA3/St tumors were generated by subcutaneous injection of 1.5 × 10⁶ TA3/St cells in 200 μl of Hanks’ balanced salt solution (HBSS). Tumor volume was estimated by V = 0.4a²b, where a is the short axis and b is the long axis of the tumor. IP TA3/St tumors were generated by injection of 2 × 10⁹ TA3/St cells IP in 400 μl of HBSS. For the Namalwa lymphoma tumor model, 6- to 8-wk-old CB17 SCID female mice were obtained from Charles River Laboratory (Wilmington, Massachusetts, United States). Then, 5 × 10⁶ Namalwa lymphoma cells were injected IP in 400 μl of HBSS. Peptide was dissolved in water, brought to 600 μg/ml of peptide, and injected IP. All animal studies were approved by the University of California, San Diego, Institutional Animal Care and Use Committee.

Histology. Mice harboring solid TA3/St tumors were injected with 650 μg of biotinylated RI-TAT-p53C peptide and sacrificed 1 h postinjection. Sections from frozen tumors were stained with VECTASTAIN Elite ABC Kit and DAB substrate per the manufacturer's instructions (Vector Laboratories, Burlingame, California, United States).

Statistical analysis. Student’s t-test was used to determine statistical significance (p < 0.05) in all experiments except animal survival experiments, in which the Wilcoxon Rank-Sum Test was performed.
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Conflicts of interest. SFD is the scientific founder of Ansata Therapeutics, a biotech company utilizing protein transduction technology to treat human disease. However, he owns less than 5% of the stock, does not receive any research funds from Ansata, does not have a seat on the board of directors, and has no say in the governance of the company.

Author contributions. ELS and SFD conceived and designed the experiments. ELS, BRM, and CCS performed the experiments. CCS and SFD analyzed the data. BRM and CCS contributed reagents/materials/analysis tools. ELS and SFD wrote the paper.