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Report

Enhanced suppression of prostate tumor growth by combining C-CAM1 gene therapy and angiogenesis inhibitor TNP-470

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We have previously shown that C-CAM1-based gene therapy effectively suppressed prostate tumor growth in nude mice xenograft models. In this study, we examined the effects of combining C-CAM1-based therapy and TNP-470, a potent angiogenesis inhibitor, on prostate cancer in a xenografted tumor model. The direct cytotoxic effects of Ad-C-CAM1 (recombinant adenovirus containing C-CAM1 cDNA) and TNP-470 on DU145 cells *in vitro* were determined by microculture tetrazolium assay. The *in vivo* antitumor effects of either agent alone were studied in a DU145 xenografted tumor model. Cells were infected with Ad-C-CAM1 or the control virus at multiplicities of infection (m.o.i.) of 5 or 10 and then inoculated onto nude mice 48 h later. TNP-470 (0, 17 or 35 mg/kg) was given 15, 17 and 19 days after inoculation. Combined treatments *in vivo* were carried out to determine whether there were synergistic antitumor effects. Both Ad-C-CAM1 and the control virus were minimally toxic to DU145 *in vitro*. There was evident dose-dependent suppression of xenografted tumor growth by either Ad-C-CAM1 or TNP-470. By the median-effect analysis, combination of the two agents generated strong synergistic antitumor effects as shown by marked tumor suppression as compared to either treatment alone. The novel strategy may have clinical implications for the treatment of prostate cancer. [© 2002 Lippincott Williams & Wilkins.]

Key words: Cell adhesion molecule, gene therapy, prostatic neoplasms, tumor suppressor, xenograft.

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Introduction

Prostate cancer is the most common non-skin cancer and the second leading cause of cancer death in men in the US. Because existing therapies are not effective for advanced prostate cancer, new therapies are urgently needed. We have identified a new target for prostate cancer therapy, the tumor suppressor cell-cell adhesion molecule 1 (C-CAM1). C-CAM1 expression is either reduced or completely lost in abnormal prostate growth, including high-grade prostatic intraepithelial neoplasia and prostate cancer.[1,2] This observation suggests that C-CAM1 may play a critical role in maintaining normal prostate function and that its disappearance may contribute to the onset of abnormal prostate growth. Consistent with this hypothesis, down-regulation of C-CAM1 expression in non-tumorigenic NbE cells using an antisense approach renders these cells tumorigenic.[3]

The therapeutic potential of this molecule has been demonstrated in several studies. We have shown that restoring C-CAM1 function by transfecting the C-CAM1 gene into PC-3 prostate cancer cells suppresses their growth.[3] In addition, introducing C-CAM1 in an adenovirus vector (Ad-C-CAM1) into prostate tumors results in significant regression of these tumors.[4,5] Preclinical studies on the efficacy of Ad-CD66a, a recombinant adenovirus containing the human C-CAM1 gene, on prostate tumors showed promising antitumor activity.[6] Although Ad-C-CAM1 treatment alone is expected to have some therapeutic effects, single-modality treatments usually have limited anticancer effect clinically. The combined use

of drugs with different mechanisms of action usually enhances the therapeutic outcomes.

The ability of tumors to grow and metastasize depends in part on their ability to induce angiogenesis.[7] Therefore, angiogenesis inhibitors may be used to control both tumor growth and metastasis. TNP-470, a synthetic analog of the fungal metabolite fumagillin, is an angiogenesis inhibitor with a great potential in cancer therapy.[8] TNP-470 was shown to have an antitumor effect on a number of malignancies, including glioma,[9] breast[10] and prostate cancer.[10,11] TNP-470 was shown to have specific cytostatic effects on endothelial cells *in vitro*.[12] TNP-470 also inhibits endothelial cell proliferation *in vitro* and tumor-induced angiogenesis *in vivo*.[8] In addition, it inhibits *in vivo* angiogenesis when administered topically to chick embryo chorioallantoic membranes or the rat cornea.[13] Studies by Sin *et al.*[14] showed that TNP-470 acts by binding to methionine aminopeptidase-2 (MetAP-2). Griffith *et al.*[15] demonstrated that inhibition of the MetAP activity by fumagillin analogs correlates with their ability to inhibit endothelial cell proliferation, suggesting that the enzymatic activity of MetAP-2 is critical for endothelial cell proliferation. These studies suggest that TNP-470 is a promising anti-angiogenic agent with a unique mechanism of action.

Because TNP-470 is a cytostatic agent for endothelial cell proliferation, complete tumor regression may not be attained by subtoxic doses of TNP-470. We hypothesized that combining C-CAM1 with TNP-470 would have a synergistic effect by targeting different points in the tumorigenesis pathways. In this study, we investigated whether the combination of C-CAM1 and TNP-470 further enhances their antitumor efficacy.

Materials and methods

Construction of recombinant adenovirus containing the C-CAM1 gene

Recombinant adenoviruses containing the full-length C-CAM1 cDNA in the sense orientation (Ad-C-CAM1) and antisense orientation (Ad-AS) to the cytomegalovirus promoter were generated as described previously.[5] Ad-AS served as a control for viral toxicity.

Microculture cytotoxicity assay

The prostate cancer cell, DU145, is an androgen-independent prostate cancer cell line established from patients with prostatic carcinoma metastasizing to the brain.[16] Previous study has shown that DU145 cells did not express C-CAM1.[6] TNP-470 was obtained from Developmental Therapeutics Program (National Cancer Institute, Bethesda, MD). The direct cytotoxic effects of Ad-C-CAM1 and TNP-470 on DU145 cells were assessed by a modified cell viability assay with MTT (Sigma, St Louis, MO).[17] In brief, 5,000 DU145 cells per well in 100 μl of culture medium were seeded into 96-well microplates and incubated at 37°C for 24 h before exposure to Ad-C-CAM1, Ad-AS or TNP-470. This number of cells plated (i.e. 5000/well) was titrated so that the cells in control wells were in the exponential phase of growth throughout the 96-h incubation period before cell viability was measured. Ad-C-CAM1, Ad-AS or TNP-470 diluted in culture medium was added to a final volume of 200 μl/well. After 72 h, 50 μl of MTT (2 mg/ml in Dulbecco's modified Eagle's medium) was added to each well and allowed to react for 2.5 h. The blue formazan crystals that formed were pelleted to the bottom of wells by centrifugation, the supernatant was removed and the precipitates were dissolved in 150 μl of dimethylsulfoxide (Sigma). The optical density was determined by absorbance spectrometry at 492 nm with a microplate reader (SLT Molecular Device, Untersbergstrabe, Austria). Three separate experiments with triplicate measurement for each treatment were performed. The IC_{50}s were calculated by median-effect analysis and expressed as means $\pm$ SD.[18]

Tumor growth *in vivo*

Male BALB/c *nu/nu* mice were obtained from Harlan Sprague Dawley (Indianapolis, IN) at 6–8 weeks of age. DU145 cells were infected *in vitro* with Ad-AS or Ad-C-CAM1 with varied multiplicities of infection (m.o.i.) and injected s.c. (2×10^6 cells/site) into the mice. TNP-470 (17 or 35 mg/kg body weight) or the vehicle was given to the mice by s.c. injection at a site remote from the tumors at 15, 17 and 19 days of tumor inoculation, making the total dose of TNP-470 around 50 and 100 mg/kg body weight. The s.c. tumors were measured weekly with a sliding caliper. Tumor size was calculated according to the method of Rockwell *et al.*[19] and expressed as mean $\pm$ SD. The effects of treatments were quantified by the formula: T/C (%) = (mean tumor size of treated group/mean tumor size of control group) $\times$ 100%.

Statistical analysis

The general mixed-model approach[20] with random effects was used to analyze the longitudinal data of

tumor growth in different treatment groups by using SAS PROC MIXED (SAS Institute, Cary, NC). The key assumption was that the data arose from a multivariate normal distribution with a linear mean model and a reasonable covariance structure. Verification of the assumptions was performed via profile plots, which showed roughly bell-shaped dispersion at each of the time points (except for the saturated treatments), with no radical outliers. The covariance structure used in the analysis modeled completely general variances and covariances for all of the observations from the same subject. Each subject was assumed to possess the same covariance structure and data from different tumors were assumed to be independent. The restricted maximum likelihood method was used to fit the mixed model, and to make inferences on significant treatment or time effects and their interactions. The data are expressed as mean $\pm$ SD.

The combined antitumor effect of Ad-C-CAM1 gene therapy and TNP-470 treatment *in vivo* was assessed by median-effect analysis with the mutually nonexclusive model described by Chou and Talalay.[18] The combined effects were indicated by the combination indexes. Combination indexes less than, equal to and greater than 1 indicate synergistic, additive and antagonistic effects, respectively.

Results

Effects of Ad-C-CAM1 and TNP-470 on the viability of DU145 cells *in vitro*

Although Ad-C-CAM1 at an m.o.i. of 10 generated significant antitumor activity *in vivo*,[21] incubation of DU145 cells with Ad-C-CAM1 or Ad-AS *in vitro* had no significant cytotoxic effect (Figure 1A). At the highest Ad-C-CAM1 m.o.i. tested (100), the percentages of Ad-C-CAM1- and Ad-AS-treated cells surviving were 97 and 96%, respectively, which indicates that these recombinant viruses had no direct cytotoxic effect on DU145 cells. No difference of cell viability was seen between Ad-C-CAM1 and Ad-AS.

By the MTT assay, the IC_{50} of TNP-470 to DU145 cells was $3.05 \pm 0.16\,\mu$g/ml (Figure 1B), which is 10^5 times greater than TNP-470's effect on human umbilical vein endothelial cells (0.015 ng/ml).[12] This result is consistent with other reports that epithelial cells are less sensitive to TNP-470 than endothelial cells.[12]

Dose-dependent antitumor activity of C-CAM1 *in vivo*

DU145 cells were infected with Ad-C-CAM1 or Ad-AS at various m.o.i. and injected s.c. into male *nu/nu* mice. As shown in Figure 2, treatment of DU145 cells with Ad-C-CAM1 at an m.o.i. of 10 completely suppressed the growth of DU145 xenografted tumors *in vivo*, whereas an m.o.i. of 5 only partially inhibited the tumor growth (T/C $\sim$ 50%) throughout the entire study period up to 6 weeks after the inoculation. Ad-AS at an m.o.i. of 10 was minimally toxic as shown by the exponential growth of xenografted tumors by 6 weeks.

Dose-dependent antitumor activity of TNP-470 *in vivo*

DU145 cells were injected s.c. into male *nu/nu* mice and allowed to grow for 2 weeks before the TNP-470 treatment. Mice carrying the DU145 xenografted tumors were treated with TNP-470 (17 or 35 mg/kg s.c.) or with vehicle only on days 15, 17 and 19. An initial reduction of tumor size was observed at 21 days in mice treated with both doses of TNP-470 (Figure 3). However, tumor growth resumed in a week after the last TNP-470 injection (4 weeks after DU145 inoculation), resulting in a tumor size reduction of 75 and 45% for 17 and 35 mg/kg TNP-470, respectively, at 42 days of tumor growth.

Antitumor activity of TNP-470 in combination with Ad-C-CAM1

The combined effect of Ad-C-CAM1 and TNP-470 on tumor growth was tested. DU145 cells infected with Ad-C-CAM1 or Ad-AS at an m.o.i. of 5 were implanted s.c. into male *nu/nu* mice. TNP-470 (35 mg/kg) or buffer was given to the mice 15, 17 and 19 days (in the third week) after tumor cell inoculation. The antitumor effect of the combined treatment was significantly greater than that of either agent alone (Figure 4). With the most effective combination of Ad-C-CAM1 and TNP-470, the maximal tumor suppressive effect was observed at 21 and 28 days of tumor inoculation (i.e. the first and second weeks after the initiation of TNP-470 treatment) in that all mice were tumor-free. Of the 12 xenografted tumors in this group, one small tumor re-appeared at 35 days and three at 42 days. However, these tumors were much smaller than those of the controls. The tumor regrowth after discontinuation of TNP-470 treatment

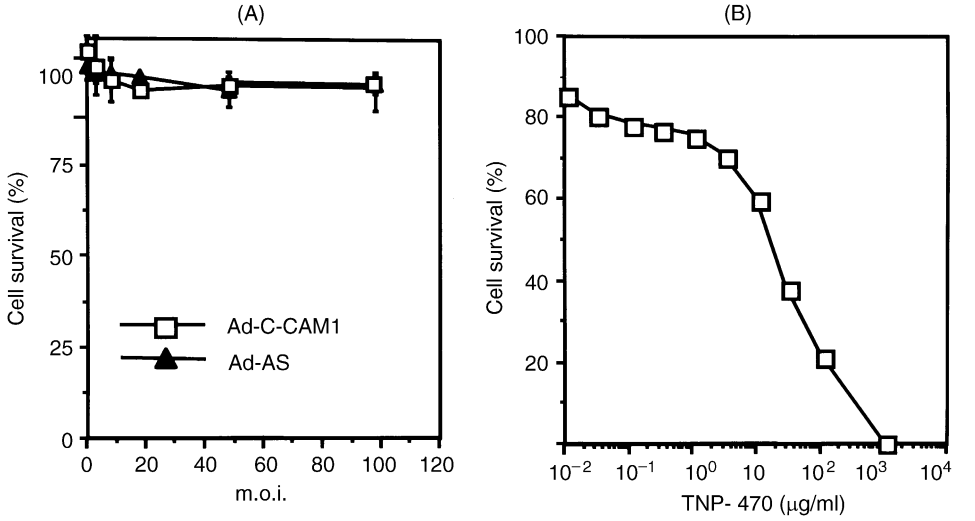

Figure 1. Effect of Ad-C-CAM1 and Ad-AS (A) and TNP-470 (B) on the growth of DU145 cells *in vitro*. No evident cytotoxic effects were seen in cells treated with either Ad-C-CAM1 or Ad-AS.

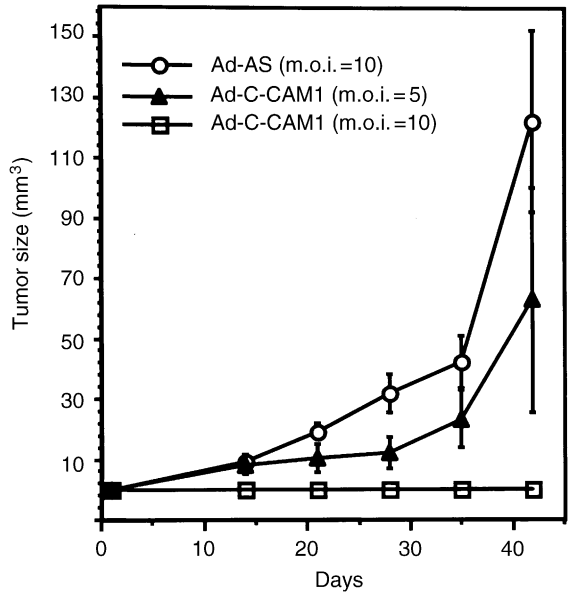

Figure 2. Dose-dependent growth inhibition of DU145 xenografted tumors by Ad-C-CAM1. DU145 cells were first infected with Ad-C-CAM1 or Ad-AS at various m.o.i. as indicated. After 48 h of incubation, 2×10^6 cells were injected s.c. into the flanks of a male nude mouse on day 0. Each data point represents the mean tumor size ± SD of 12 tumors.

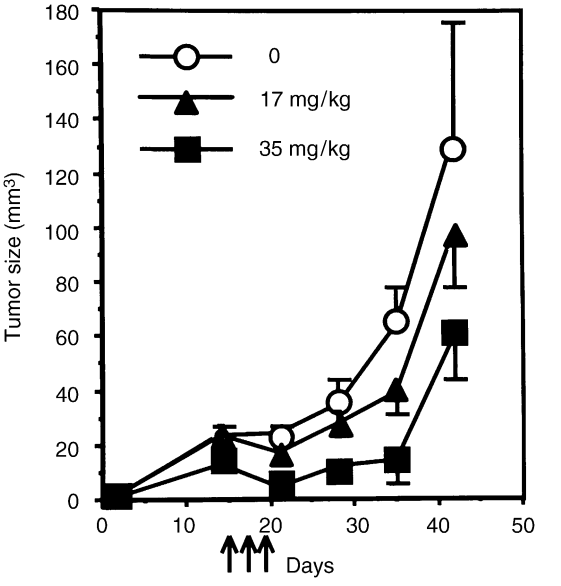

Figure 3. Inhibition of DU145 tumor growth by TNP-470. DU145 cells (2×10^6) were injected s.c. into the flanks of a male nude mouse on day 0. TNP-470 was administered s.c. on days 15, 17 and 19. Each data point represents the mean tumor size ± SD of eight tumors.

is consistent with the previous observation that TNP-470 has a short-lived cytostatic effect on endothelial cells.[12] At 42 days, the tumors in the combined treatment group averaged about 6 mm^3, whereas those in the Ad-AS and Ad-C-CAM1 alone groups averaged 123 and 63 mm^3, respectively (Figure 4).

The mixed-model analysis showed significantly slower tumor growth in the combined treatment group than in the Ad-AS group ($p < 0.001$) throughout the entire course of the study. Significant differences were also obtained between the combined treatment group and the Ad-AS plus TNP-470 ($p = 0.01$) and Ad-C-CAM1 alone groups ($p < 0.03$) at 21 and 28 days. With the median-effect analysis, the

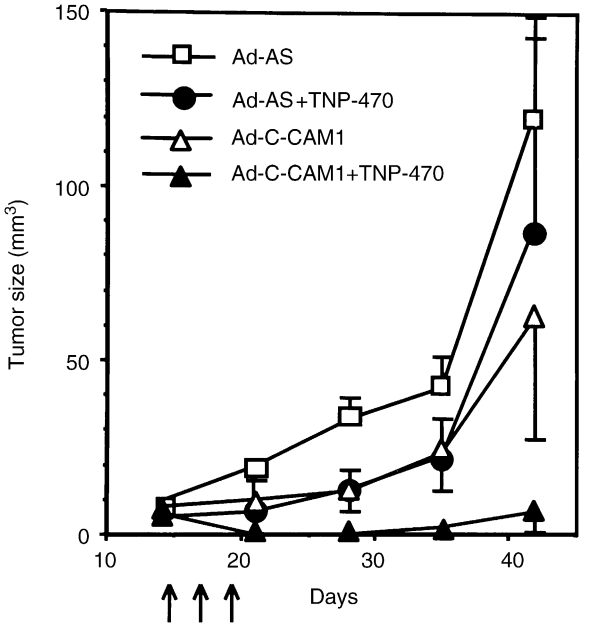

Figure 4. Antitumor activity of Ad-C-CAM1 plus TNP-470. DU145 cells were infected with Ad-C-CAM1 or Ad-AS at an m.o.i. of 5. After 48 h of incubation, 2×10^6 cells were injected s.c. into the flanks of male nude mice on day 0. TNP-470 or the vehicle only was administered s.c. on days 15, 17 and 19. Each data point represents the mean tumor size $\pm$ SD of 12 tumors.

combination indexes of the combined treatment were 0.047 and 0.331 at 21 and 28 days, respectively, indicating that Ad-C-CAM1 and TNP-470 interact synergistically to suppress DU145 tumor growth in this model. As tumors regrew in the fifth week, the combination index increased to 1.092, indicating that the combined effect declined from synergistic to only additive.

Discussion

Advanced prostate cancer is still an incurable disease. Novel therapeutic strategies that can effectively treat advanced prostate cancer will have a major impact on this disease. We have described in this study a novel combination therapy of C-CAM1-based gene therapy and an angiogenesis inhibitor, TNP-470, for advanced prostate cancer in a mouse xenograft model. Although in the *in vitro* assay, Ad-C-CAM1 had no direct effect on tumor cell viability (Figure 1A) and TNP-470 is far less cytotoxic to epithelial cells than to endothelial cells (Figure 1B), combining these two agents proved highly effective in suppressing xenografted tumor growth *in vivo*. Median-effect analysis showed that these two agents act synergistically *in vivo*. These results suggest that restoring the expression of C-CAM1 in DU145 cells in combination with TNP-470 treatment markedly suppressed the malignant phenotype of prostate cancer cells and that the novel strategy may have clinical implications for the treatment of advanced prostate cancer.

TNP-470 inhibits the growth of endothelial cells that are actively replicating but not those in a quiescent state. Growth suppression by TNP-470 was thought to result from cytostatic angiogenesis inhibition.[12] Because TNP-470 is a cytostatic agent for endothelial cell proliferation, complete tumor regression could not be attained by subtoxic doses of TNP-470, as is the case with other anti-angiogenic modulators and biological agents. In this study, tumors resumed growth within 2 weeks after the last TNP-470 injection (Figures 3 and 4). Loss of synergism in the combined treatment group was also apparent 2 weeks after TNP-470 treatment was terminated. Therefore, the tumor-suppressive effects of the combination might be improved by increasing the TNP-470 dosing frequency or continuing TNP-470 treatment throughout the entire study period. In any event, we have shown that a strong synergism can be achieved with C-CAM1 gene therapy and short-term TNP-470 treatment.

That Ad-C-CAM1 can suppress the tumor growth and induce the regression of advanced prostate cancer strongly suggests that C-CAM1 play a critical role in the pathways that regulate the progression of this disease. Although the mechanism by which C-CAM1 inhibits tumor growth is not clear, we have shown that expression of C-CAM1 had no direct effect on tumor cell viability *in vitro* (Figure 1). Thus, the antitumor activity *in vivo* was much greater than that anticipated by the *in vitro* studies (Figures 1 and 2). On the basis of these observations, we hypothesize that C-CAM1 may function by modulating tumor cell host environments, typically by affecting stroma–epithelia interactions. As TNP-470 acts by inhibiting angiogenesis that also affects tumor cell environment, further modulation of the tumor–host microenvironment by C-CAM1 may explain the synergistic antitumor effect *in vivo*.

Induction of drug resistance in tumor cells is a major problem for patients undergoing chemotherapy. In addition, prostate tumors are intrinsically resistant to most antitumor drugs. These factors are a great challenge for clinicians in prostate cancer treatment. Agents that do not act directly on cancer cells might be more effective for the treatment of cancers with intrinsic or acquired drug resistance. For example, angiogenesis inhibitors such as TNP-

470 can prevent cancer cells from getting the necessary nutrients. Similarly, strategies like restoring C-CAM1 expression that do not exert direct cytotoxic effect on tumor cells are also promising. Combination therapies that use agents acting with similar but not identical mechanisms might produce synergistic effects, as shown in this study. This approach should be applicable to other cancers in which drug resistance is a common obstacle to effective therapies.

The standard regimen for metastatic prostate cancer is androgen ablation. Despite initial responsiveness, tumor cells inevitably relapse into an androgen-independent state. The mortality associated with prostate cancer is mostly due to recurrent androgen-independent disease, for which effective therapeutic regimens are lacking. C-CAM1-based therapy has been shown to be effective in the treatment of androgen-independent prostate tumors.[4–6] In previous studies, TNP-470 has also been shown to inhibit the *in vivo* growth and metastasis of rat[11] and human[10] androgen-independent prostate cancer. The synergistic effect of C-CAM1 and TNP-470 should be explored further, and may represent a novel approach in the treatment of both androgen-dependent and -independent prostate cancer. In conclusion, Ad-C-CAM1 has no direct cytotoxic effect on prostate cancer cells and TNP-470 is far less cytotoxic to epithelial cells than to endothelial cells. Combining these two agents proved to be a synergistic combination and is highly effective in suppressing xenografted tumor growth *in vivo*. These results suggest that restoring C-CAM1 expression in DU145 cells in combination with TNP-470 treatment markedly suppressed the malignant phenotype of prostate cancer cells and that the novel strategy may have clinical implications for the treatment of advanced prostate cancer.

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