Low-Dose Combretastatin A4 Phosphate Enhances the Immune Response of Tumor Hosts to Experimental Colon Carcinoma

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Abstract Purpose: Although there is a need to enhance the therapeutic efficiency in cancer by combining immunotherapeutic procedures with other therapy, combination with chemotherapy is complicated due to immunosuppressive effects of most chemotherapeutic drugs. The purpose of this investigation was to study whether combining tumor cell immunization with the vascular targeting drug combretastatin A4 phosphate (CA4P) would enhance tumor retardation and/or affect the antitumor immune response.

Experimental Design: Rats with intrahepatic colon carcinoma were immunized weekly with IL-18/IFNγ-transfected tumor cells, starting day 9, and were treated with a low-dose CA4P (2 mg/kg, 5 days a week starting day 7). The effect of CA4P was studied on tumor growth and on immune reactivity in vitro.

Results: Rats with preexisting tumor, immunized and treated with low-dose CA4P, had a significantly retarded tumor growth compared with rats receiving CA4P or immunization alone. Splenocytes from rats treated with this combination had a significantly enhanced antitumor immune response compared with splenocytes from control rats. Exposure of nonadherent splenocytes to CA4P in vitro did not enhance their proliferation. However, 3-hour pretreatment of adherent splenocytes with 0.3 μg/mL CA4P significantly enhanced proliferation and IFNγ production of admixed nonadherent splenocytes, partly due to nitric oxide reduction. Combining the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester with CA4P and immunization further retarded tumor growth.

Conclusion: Concomitant treatment of rats with progressively growing tumor with immunization and low-dose CA4P significantly enhances the therapeutic effect as compared with either treatment alone and results in an enhanced antitumor immune reactivity.

Active immune therapy against cancer has been developed in several animal models using whole tumor cells genetically modified to produce immune stimulatory cytokines, including IFN-γ, IL-12, and IL-18 (1–4). The aim has been to enhance the proliferation and activation of antitumor effector T lymphocytes. Efficient effector cells can be induced and a delayed tumor outgrowth can be achieved. Immunosuppression observed in cancer patients (5) and in tumor-bearing experimental animals (6–8) is a likely reason why complete eradication of tumors is rare despite the expression of tumor antigens. Various mechanisms for the generation of immunosuppression have recently been reviewed (9). There is consequently an obvious need of combining immunization with treatments that can reduce the tumor mass so as to limit its capacity to induce immunosuppression and diminish the remaining tumor burden. Several therapies can indeed reduce the tumor size or cause its temporary disappearance, but these therapies are often incompatible with simultaneous immunization because they are themselves immunosuppressive.

The vascular targeting drug combretastatin A4 phosphate (CA4P) inhibits tumor growth by selectively damaging tumor vasculature (10), which causes up to 100-fold decrease in tumor blood flow (11), leading to tumor starvation. After dephosphorylation, CA4P binds near or at the colchicine binding site on tubulin (12), causing destabilization of tubulin polymers of the cytoskeleton (13). In contrast to most other drugs used in tumor chemotherapy, CA4P does not cause any detectable lymphopenia or depletion in lymphoid organs and, as far as tested, does not suppress immune reactivity,3 making it more suitable for simultaneous combination with immunotherapy.

In the present investigation, we studied the feasibility of combining CA4P therapy with tumor immunization in a rat colon carcinoma model. It has previously been shown in this model that immunization of tumor-free animals with IL-18- and IFNγ-transfected tumor cells induces a strong T-cell

3 Sjögren et al., unpublished findings.
response towards syngeneic tumor cells demonstrable in vitro (8). As shown by in vitro techniques, immune responses can also be induced in animals already bearing a growing tumor, although the response is weaker and does not result in any detectable retardation of tumor growth, presumably due to the immune suppression developing during the progressive growth of the tumor. An important mechanism of this immune suppression is the production of excessive amounts of nitric oxide by adherent cells including macrophages and dendritic cells. Although nitric oxide at a low concentration is required for normal T-cell functions (14, 15), high concentrations lead to peroxynitrite formation, which is strongly suppressive on T-cell functions (16, 17). In vitro, the suppressive effect induced by the adherent cells can be counteracted by the competitive nitric oxide synthase inhibitor N-nitro-l-arginine methyl ester (l-NAME; refs. 7, 8). In the present study, we discovered an enhanced inhibitory effect on tumor growth when combining low-dose CA4P and immunization with tumor cells producing IL-18 and IFNγ. We show that CA4P enhances immune responses to tumor indirectly by reducing one of the immunosuppressive mechanisms in the tumor model used.

Materials and Methods

Animals. Rats of the F1 cross between the strictly inbred strains Brown Norwegian (BN) and Wistar/Furth (W/F) were used. They were bred by continuous brother-sister mating in the breeding facilities of the Biomedical Center Animal facility, University of Lund. All animal procedures are in agreement with the ethical rules of the Swedish Board of Animal Research.

Tumor cells and the IL-18/IFNγ transfectants. The rat tumor cell line H1D2 wild-type (H1D2-WT) was established from a colon carcinoma BN7005 induced by 1,2-dimethylhydrazine in a BN rat (18). The cells were cultured in vitro in RPMI 1640 supplemented with 10% FCS, 2 mM/L L-glutamine, 10 mM/L HEPES, 0.5 mM/L pyruvate, and 0.096% NaHCO3 (RPMI-FCS). For restimulation of lymphocytes with tumor cells, H1D2-WT or IL-18/IFNγ double-transfected clone of the H1D2 colon cancer cells (H1D2-IL-18/IFNγ) was established as previously reported (8). For selection of transfected cells, the selective medium containing G418 (16 μg/mL, Life Technologies, Inc., Paisley, United Kingdom) was used. The G418 was removed 1 week before transfected cells were used for immunization. The cells used for immunization were irradiated with 70 Gy from a 137Cs source before use.

Generation of intrahepatic tumor bearers. Cultured H1D2-WT cells were suspended in medium and 1 × 10⁵/50 μL were injected intrahepatically beneath the liver capsule. This dose has previously been shown to result in development of a macroscopically visible tumor within 1 week. After 4 weeks, all animals were laparatomized and tumor sizes were recorded. The longest diameter (a) and the one perpendicular to this (b) were measured by caliper, and tumor volume was calculated according to the formula 0.4 × a × b × b (derived empirically by weighing measured tumors in rats).

Treatment and immunization. Drug treatment was initiated on day 7, when the tumor would be already macroscopically visible as a small nodule. CA4P (Oxigene Europe AB, Lund, Sweden), 2 or 5 mg/kg, as specified, was administered i.p. 5 days a week. l-NAME (Sigma Chemicals Co., St. Louis, MO) was given orally, 12 mg twice a day, thrice a week. Rats were immunized i.p. at days 9, 15, and 23 (Fig. 1B) or days 10, 17, and 24 (Figs. 1A and 5) after tumor inoculation with 3 × 10⁶ H1D2-IL-18/IFNγ-irradiated cells.

Spleen cell preparation. Spleens were removed 1 week after the last immunization and were suspended as previously described (19). These cells (i.e., the whole spleen cell population) are referred to as splenocytes (SPLC). Adherent cells were removed by incubation in plastic culture bottles for 2 × 45 minutes at 37°C, generating the nonadherent SPLC population. All spleen cell populations were suspended in RPMI-FCS with 50 μmol/L l-mercaptoethanol.

Proliferation assay. For restimulation of lymphocytes with tumor cells, H1D2-WT or IL-18/IFNγ tumor cells were irradiated (70 Gy) and incubated in 96-well plates (1.5 × 10⁶/mL overnight at 37°C in a humidified 5% CO2 atmosphere. Spleen cells (SPLC or nonadherent SPLC) were added to the 96-well plate (3 × 10⁵ per well) and cultured for 5 days with the stimulatory tumor cells.

Adherent spleen cells of tumor-bearing rats were obtained by allowing 6 × 10⁵ spleen cells (not previously subjected to plastic adherence) to adhere for 2 hours in 96-well plates, after which the nonadherent cells were removed. The adherent cells were treated with various doses of CA4P as specified or with medium only. After 3 hours, the drug was washed away. Nonadherent cells were added to the plates and incubated for 5 days. Proliferation of nonadherent SPLCs cocultured with adherent SPLC was pretreated for 3 hours with CA4P at 0.3 μg/mL was compared with the proliferation of the nonadherent SPLC cocultured with medium incubated adherent SPLC. Six hours before harvesting the cell culture, [3H]thymidine was added to measure the proliferative response. The cells were harvested on filter papers, scintillation fluid was added, and the radioactivity was determined in a scintillation counter (Wallac Microbeta, Turku, Finland). Data are presented as counts per minute (cpm; Figs. 2 and 3B-C).

To investigate the direct effect of CA4P on T cells, the nonadherent SPLCs were incubated for 3 hours with CA4P at 1.5 and 0.15 μg/mL in 10-mL Falcon tubes at 37°C. The cells were washed thrice and added to the 96-well plates precoated with stimulatory tumor cells H1D2-WT and IL-18/IFNγ. The proliferative response was measured as described above using five replicates per sample. Data are presented as stimulation index (Fig. 3A). Stimulation index = cpm (nonadherent SPLC + CA4P) / cpm (nonadherent SPLC – CA4P).

Cytokine production assay. Supernatants from restimulated SPLC cultures were collected at the end of the culture period and stored at –20°C. The amounts of IFN-γ or IL-10 were measured from duplicate wells using the cytometric bead array system (BD Pharmingen, San Jose, CA).
samples using an ELISA kit (OptEIA Set, PharMingen, San Diego, CA) according to the instructions of the manufacturer.

**Measurement of nitric oxide as nitrite production.** Supernatants of SPLC cultures, stimulated either with irradiated tumor cells H1D2-WT for 5 days or with the supernatant staphylococcal enterotoxin A at 0.1 ng/mL (Toxtech, Madison, WI) for 3 days in the presence of various concentrations of CA4P (0.015, 0.03, 0.15, and 1.5 μg/mL for 3 hours), were added to 96-well plates and nitric oxide production was measured as nitrite \( \text{NO}_2^- \) concentration by the Griess assay (20).

**Assessment of viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay.** The effect of CA4P on cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (21). Splenocytes \((6 \times 10^5)\) were allowed to adhere for 2 hours in a 96-well plate. After removal of nonadherent cells, the adherent SPLC were incubated with 0, 10, 30, 100, 300, 1,000, and 3,000 ng/mL CA4P for 3 hours, and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-assay was done 3, 20, 45, and 65 hours after CA4P removal. Four animals were included at each time point and the results are presented as the mean of five replicate wells per CA4P concentration.

**Statistics.** We did statistical evaluation using StatView software. When data followed the Gaussian distribution, significance of differences among three or more groups was estimated by ANOVA, followed by the Tukey-Kramer test for pairwise comparisons. When data did not follow Gaussian distribution, the Kruskal-Wallis nonparametric ANOVAs was used for evaluation of differences among three or more groups and Mann-Whitney U test for differences between two groups. \( P < 0.05 \) (two tailed) was considered statistically significant.

**Results**

Significantly enhanced retardation of tumor growth by a combination of low-dose CA4P \((2 \text{ mg/kg})\) and tumor immunization as compared with CA4P or immunization alone. It has previously been reported that the vascular targeting drug CA4P at doses of 30 to 100 mg/kg inhibits tumor growth in rats by selectively damaging tumor neovasculature (11). In rats bearing an intrahepatic wild-type tumor, immunization with tumor cells expressing IL-18 and IFN-\( \gamma \) does not result in any detectable inhibition of the tumor growth as compared with untreated rats (Fig. 1A). To enhance the therapeutic efficiency, we combined immunization with CA4P at a 15-50 times lower dose \((2 \text{ mg/kg})\) than previously used (11, 22). This combination resulted in a significantly enhanced retardation of tumor growth compared with no treatment or CA4P alone \((P = 0.0004)\) by Kruskal-Wallis and \( P < 0.05 \) by Mann-Whitney U test; Fig. 1B), whereas CA4P alone did not significantly retard tumor growth.

Low-dose CA4P enhances the immune response of tumor-bearing rats to immunization with tumor cells producing IL-18 and IFN-\( \gamma \). To address mechanisms of the enhanced tumor retardation by CA4P combined with immunization, we investigated the effect of CA4P on the efficacy of antitumor immunization by studying the immune responses using spleen cells from CA4P treated animals. Spleen cells from tumor-bearing rats immunized with IL-18/IFN-\( \gamma \)-expressing tumor cells alone, or combined with 2 mg/kg CA4P, were tested \textit{in vitro} for a proliferative response towards H1D2-WT stimulator cells. A significantly enhanced proliferation \((P < 0.05, \text{Mann-Whitney U test})\) was observed with splenocytes from rats receiving the combined treatment as compared with splenocytes from rats subjected to immunization alone (Fig. 2). A stronger proliferation was observed when adherent spleen cells were excluded both in the group receiving combined treatment and in the group only immunized, but the former group showed a significantly higher proliferation \((P < 0.05, \text{Mann-Whitney U test})\).

Exposure of adherent spleen cells to CA4P reduces their suppressive effects. We first investigated whether the elevated proliferative response of splenocytes is due to a stimulatory effect of CA4P directly on T cells. A 3-hour treatment with 0.15 or 1.5 μg/mL CA4P had no stimulatory effects on the proliferative response of the nonadherent spleen cells, neither when restimulated with H1D2-WT nor with H1D2-IL-18/IFN-\( \gamma \) tumor cells. Instead, although not significant, CA4P seemed to diminish significantly \((P < 0.05, \text{Mann-Whitney U test})\) their suppressive effect on the proliferation of the nonadherent spleen cells, neither when restimulated with H1D2-WT nor with H1D2-IL-18/IFN-\( \gamma \) tumor cells. Instead, although not significant, CA4P seemed to slightly inhibit lymphocyte proliferation at the doses tested (Fig. 3A).

Because adherent spleen cells of tumor-bearing rats have previously been shown to suppress the response of admixed T lymphocytes (7, 8), we investigated the \textit{in vitro} effect of CA4P on this suppression. Exposure of adherent spleen cells for 3 hours to CA4P at a concentration as low as 0.3 μg/mL diminished significantly \((P < 0.05, \text{ANOVA})\), and then Tukey-Kramer test for pairwise comparison) their suppressive effect on the proliferative response of admixed nonadherent SPLC towards tumor cells \(\text{H1D2-WT or H1D2-IL-18/IFN-\( \gamma \)}} \) tumor cells. Instead, although not significant, CA4P seemed to slightly inhibit lymphocyte proliferation at the doses tested (Fig. 3D).

CA4P acts partly by reducing the nitric oxide–mediated suppression. The possible effect of CA4P on the nitric oxide–mediated suppression previously reported in the model used (6, 7) was studied by incubation of spleen cells for 3 hours with various concentrations of CA4P. After 3 days of proliferation with staphylococcal enterotoxin A, the nitric oxide concentration was reduced by 50% to 60% in the presence of CA4P (Fig. 4A). This reduction was observed at all concentrations used. Even at concentrations as low as 0.015 μg/mL, a similar reduction was shown, indicating that the decreased nitric oxide...
production was not caused merely by an effect on cell viability. This effect was also obtained when splenocytes were restimulated with H1D2-WT cells. We also analyzed which concentration of CA4P had a direct effect on the viability of the adherent SPLC. Adherent splenocytes were treated for 3 hours with CA4P. After removal of CA4P and incubation of the cells for 3 to 65 hours at 37°C, the cell viability was measured.

No toxicity was observed after 3 hours at doses of CA4P up to 3 µg/mL (Fig. 4B). After 20 to 65 hours, doses up to 1 µg/mL had no significant effect on viability, whereas the highest dose of 3 µg/mL caused a 40% to 50% reduction of cell viability. The lowest dose showing cytotoxicity is thus 3 µg/mL, which is 30 times higher than the one that reduced nitric oxide concentration down to 50%. This indicates that CA4P is not cytotoxic for adherent splenocytes at relevant doses.

Spleen macrophages of tumor-bearing hosts have been reported to cause immune suppression in other models, including a release of IL-10 (23, 24). We therefore investigated the effect of CA4P on IL-10 production from splenocytes. IL-10 levels were not affected by a 1.5-hour treatment of adherent SPLC with 0.3 µg/mL CA4P (Fig. 4C).

Comparison of the inhibitory effect on tumor growth in vivo by CA4P and the nitric oxide synthase inhibitor L-NAME. Because CA4P may act partly by decreasing the nitric oxide production in the splenocyte culture supernatant as described above, we compared the CA4P-induced tumor retardation in immunized rats with that caused by L-NAME, a nonselective nitric oxide synthase inhibitor. Both CA4P (5 mg/kg 5 times a week starting on day 7) and L-NAME significantly retarded tumor growth to about the same extent at the dose used compared with immunization alone (P < 0.001, Kruskal-Wallis test). Moreover, the combination of CA4P with L-NAME resulted in a significantly further augmentation of the tumor retardation (P < 0.05, Mann-Whitney test; Fig. 5).

Fig. 3. A, the proliferative response of the nonadherent SPLC treated for 3 hours with CA4P at 0.15 and 1.5 µg/mL, respectively, and restimulated with either wild-type tumor cells (H1D2-WT) or IL-18/IFNγ transfected tumor cells (H1D2-IL-18/IFNγ). Stimulation index = cpm (nonadherent SPLC + CA4P) / cpm (nonadherent SPLC – CA4P). Columns, mean stimulation index of four rats per group; bars, SE. There are no significant differences between groups (ANOVA). B and C, the proliferative response of nonadherent SPLCs, challenged in vitro with either wild-type tumor cells (B) or IL-18/IFNγ tumor cells (C) in the presence of adherent SPLC preincubated for 3 hours with 0.3 µg/mL CA4P or not exposed to CA4P. Columns, mean cpm values of five parallel samples; bars, SE. Incubation time before adding [³H]thymidine was 5 days. Representative of four experiments. There are significant differences between groups (P < 0.0001, ANOVA). *, P < 0.05, significant difference between nonadherent cells with admixed adherent cells treated with CA4P versus adherent cells unexposed to CA4P (Tukey-Kramer test). D, IFNγ (pg/mL) production from nonadherent SPLC cocultured with adherent splenocyte, either preincubated with 0.3 µg/mL CA4P for 1.5 hours or not exposed to CA4P. Cells were restimulated for 5 days with wild-type tumor cells (H1D2-WT) and IL-18/IFNγ–transfected tumor cells, respectively. Columns, mean; bars, SE. **, significant difference between groups of CA4P-exposed and nonexposed adherent cells (P < 0.005, ANOVA; P < 0.05, pairwise comparison by Tukey-Kramer test).
Discussion

In a study using CA4P to retard tumor growth by impairing the tumor vasculature in combination with immunization with tumor cells producing IL-18 and IFNγ, we discovered significantly enhanced tumor retardation by the combined treatment. Immunization alone did not, in this model, inhibit the growth of tumors already present at the time of immunization, despite inducing an initially strong immune response shown by in vitro assays. We found that CA4P, even at doses too low to induce a visible decrease of tumor vascular density (data not shown), and only slightly retarding tumor growth in vivo, caused a significantly increased inhibition of tumor growth when combined with immunization with tumor cells producing IL-18/IFNγ (Fig. 1B). The lack of therapeutic effect by immunization alone was presumably due to a pronounced systemic immunosuppression that has been shown to develop in the tumor bearers (7, 8). In the rat tumor model used, an important part of the immunosuppression is mediated by nitric oxide produced by adherent spleen cells (8). Spleen cells of immunized rats treated also with low dose of CA4P were shown to exhibit a stronger proliferative response to tumor cells (Fig. 2). Direct in vitro exposure of nonadherent spleen cells to low doses of CA4P did not strengthen their responsiveness to tumor cells (Fig. 3A). However, a similar exposure of adherent spleen cells for 3 hours reduced the suppressive effect of these cells on the proliferative antitumor responsiveness of admixed spleen lymphocytes (Fig. 3B and C). This was also indicated by the increased IFNγ levels that were detected in the SPLC/tumor cell coculture, when adherent splenocytes were exposed to CA4P (Fig. 3D).

This effect of CA4P is accompanied by a 50% reduction of nitric oxide released to the culture medium from splenocytes treated with various, relevant concentrations of CA4P, low enough not to mediate any direct cytolysis on adherent spleen cells (Fig. 4A and B). No effect was observed on the release of IL-10 (Fig. 4C). The effect on tumor growth by immunization in combination with the nitric oxide synthase inhibitor, 1-NAME, is similar to that of CA4P, and the combination of the two drugs resulted in significantly additive effects (Fig. 5).

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**Fig. 4.** A, effect of CA4P treatment for 3 hours on the nitric oxide (NO) production from splenocytes. The nitrate concentrations in the culture supernatants was analyzed by Griess assay at day 3 or 5 of culture. Splenocytes were either stimulated with 0.1 ng/mL staphylococcal enterotoxin A (SEA) for 3 days or with H1D2-WT cells for 5 days. Columns, mean nitric oxide reduction (%) of eight animals; bars, SD. B, survival of the adherent splenocytes after treatment with various concentrations of CA4P for 3 hours. The cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The viability was measured 3, 20, 45, and 65 hours after CA4P removal. Viability is recorded in percent compared with medium-treated cells. Points, mean of four animals at each CA4P concentration; bars, SD. C, IL-10 (pg/mL) production from nonadherent SPLC cocultured with adherent SPLC pretreated with 0.3 µg/mL CA4P for 1.5 hours. Cells were restimulated with staphylococcal enterotoxin A (0.1 ng/mL) for 3 days or with wild-type tumor cells (H1D2-WT) for 5 days.
It is still unclear whether CA4P acts on adherent splenocytes only by decreasing their nitric oxide production and the resulting immunosuppression in vivo or if CA4P also may improve their antigen presentation, costimulatory effect, or cytokine production in as yet undefined ways resulting in an augmented activation of effector T cells. At dose levels higher than used here, CA4P has been shown to induce apoptosis in proliferating endothelial cells in vitro (25), which might be one of the mechanisms resulting in tumor retardation. At lower dose levels, CA4P may affect certain endothelial functions leading to increased permeability of the endothelial cell layer (26), which might increase infiltration of activated immune cells, and in turn may result in an enhanced efficiency of simultaneous immunization. In view of these different effects of CA4P depending on the dose level, it will be important to determine the dose level of CA4P that gives an optimal therapeutic effect when combined with tumor immunization or other modes of immunotherapy.

In conclusion, our results in a rat colon cancer model showed that whereas tumor immunization alone was unable to induce a retardation of preexisting tumors, it caused a significant inhibition of tumor growth when combined with the anti-vascular drug CA4P. This enhanced effect was significant both as compared with rats immunized alone and with those treated with CA4P alone. The rats subjected to combined treatment showed an enhanced T-cell responsiveness as shown in vitro. Although no stimulatory effect of CA4P could be detected when lymphocytes were directly exposed to CA4P in vitro, the drug was shown to reduce the immune suppressive activity of adherent spleen cells, at least partially by reducing the excessive production of nitric oxide as shown in vitro. Whether CA4P mediates its effects only by decreasing macrophage/dendritic cell–mediated immunosuppression, or by enhancing lymphocyte trafficking via modulation of endothelial cell cadherin (26) and facilitating lymphocyte infiltration into the tumor, is currently being investigated.

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**References**

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