Captopril, an Angiotensin-Converting Enzyme Inhibitor, Promotes Growth of Immunogenic Tumors in Mice

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Abstract

Purpose: Antitumor potential of angiotensin-converting enzyme inhibitors has been shown in different preclinical settings, which always involved immunocompromised organisms or nonimmunogenic tumor models. In our study, we wanted to evaluate the effect of captopril on growth of immunogenic tumors in immunocompetent animals.

Experimental Design: We used different murine tumor models to evaluate the effect of captopril on tumor take and survival of tumor-bearing immunocompetent and immunocompromised mice. We used an orthotopic renal cell cancer model and highly immunogenic tumor model, which were based on kidney subcapsular injection of RenCa cells or s.c. injection of MethA cells, respectively. To show the influence of captopril on antigen-specific immune responses, we have used two model antigens (green fluorescent protein and β-galactosidase).

Results: Captopril decreased survival of RenCa-bearing, immunocompetent mice in a dose-dependent manner and in adjuvant setting. In nephrectomized mice, captopril shortened their survival. Captopril promoted formation of immunogenic MethA sarcoma tumors but had no effect on nonimmunogenic melanoma cells (B78-H1). Treatment of immunocompromised mice bearing MethA tumors or RenCa kidney tumors with captopril did not affect tumor formation nor survival, respectively. Captopril-treated mice immunized with AdLacZ or AdGFP vectors did not generate or generated decreased numbers of antigen-specific CD8+ T cells, respectively. However, they showed B-cell responses represented by infiltration of MethA tumors with activated B cells and dramatically increased serum level of β-galactosidase-specific antibodies.

Conclusions: Our results show a novel role of captopril in tumor biology and the tumor-promoting properties of captopril seem to be associated with its immunomodulatory potential.

Captopril (D-3-mercapto-2-methylpropanoyl-L-proline), an orally active inhibitor of angiotensin-converting enzyme (ACE) that converts angiotensin I into angiotensin II, is widely used in the management of hypertension and heart failure (1). In addition to its ability to reduce blood pressure, captopril has also several other biological activities. It can ameliorate arthritis (2), reverse diabetic retinopathy (3), enhance insulin sensitivity (1), lower thrombotic risk (4, 5), decrease atherosclerosis and renal failure (6–8), and lower the incidence of radiation-induced pulmonary damage and radiation-induced fibrosarcomas in rats (9, 10).

Moreover, ACE inhibitors are believed to decrease the risk of cancer due to reduction of angiotensin II activity, which in experimental studies was shown to stimulate neoangiogenesis by increasing vascular endothelial growth factor production (11–14), potentiate the mitogenic effects of epidermal growth factor (15), and induce cellular oncogenes c-myc, c-fos, c-N-ras, and growth factors, such as transforming growth factor-β (16, 17).

A retrospective cohort study of 5,207 patients with a follow-up of 10 years showed that long-term use of ACE inhibitors may protect against cancer, especially lowering the relative risk of gynecologic cancers (18). Long-term captopril intake decreased also the risk of prostate cancer (19). Experimental studies in vivo showed that captopril inhibited tumor growth and extended survival of tumor-bearing mice by inhibition of angiogenesis and tumor metastatic potential (20). However, antitumor potential of ACE inhibitors has only been shown in immunocompromised organisms or nonimmunogenic tumor models. Captopril is also known to display significant immunomodulatory functions (21).

In our study, we evaluated the efficacy of captopril in treatment of immunogenic tumors and showed that it promoted growth of these tumors and significantly impaired generation of functional antigen-specific CD8+ T cells.

Materials and Methods

Animals. Female BALB/c, C57BL/6, and C.B-17 scid/scid F1 mice, ages 8 to 12 weeks, were used. BALB/c and C57BL/6 mice were
purchased from the Polish Academy of Sciences (Warsaw, Poland) and C.B-17 scid/scid mice were obtained from Harlan UK Ltd. (Oxon, United Kingdom). Animals were kept under constant pathogen-free conditions in rooms with 12-hour day/night cycle with unlimited access to food and water. All experiments were done according to the guidelines approved by the Ethical Committee at the University of Medical Sciences (Poznan, Poland).

**Tumor cells.** Highly immunogenic murine RenCa (renal cell carcinoma) cells, MethA (cholanthrene-induced sarcoma) cells, and low immunogenic B78-H1 melanoma cells were used throughout experiments. Cells were maintained in DMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics, and 2 mmol/L L-glutamine (all from Invitrogen), hereafter called culture medium. Cells were cultured in 78 cm² culture plates at 37°C in a fully humidified atmosphere of 5% CO₂/95% air and passed every 3 to 4 days.

**Peptides.** The H-2Kd-restricted β-galactosidase 96-103 (DAPITVYN) CTL epitope peptide (22) and an unrelated, H-2Kd-restricted, control peptide (VMCYSSPL; ref. 23) were synthesized by Polgen (lodz, Poland). The H-2Kd-restricted enhanced green fluorescent protein (EGFP) 200-208 (HYLSTQSAL) CTL epitope peptide was synthesized by ProImmune Ltd. (Oxford, United Kingdom; ref. 24).

**Adenoviral recombinants.** An E1-deleted adenoviral recombinant of the human strain 5 expressing β-galactosidase, termed AdLacZ, has been purchased from Invitrogen. An E1-deleted adenoviral recombinant of the human strain 5 expressing EGFP, termed AdGFP, was kindly provided by Dr H. Ertl (Wistar Institute, Philadelphia, PA). The viruses were propagated and titrated on E1-transfected 293 cells as described previously (25).

**In vivo experiments.** All study groups comprised eight animals. All experiments were repeated thrice. MethA and B78-H1 cells were inoculated s.c. into left hip of mice on day 0 (5 × 10⁷ cells suspended in 100 µL PBS).

Mice (BALB/c or C.B-17 scid/scid) receiving renal subcapsular injection of RenCa cells were anesthetized with Avertin anesthesia according to a standard protocol (26). Briefly, mice received i.p. injection (0.7 mg/g) of Avertin working solution (2-2-2 tribromoethanol diluted in tert-amyl alcohol). Skin of the anesthetized mice at left lumbar region was shaved with an electric shaver. Next, the skin and s.c. tissue were cut with a scalpel and the left kidney was exposed. The wound was closed with two to three surgical stitches. In a set of experiments, 10 days after tumor implantation, mice were again anesthetized, the tumor-bearing kidney was exposed, and following ligation of renal artery and vein a nephrectomy was done according to uro- oncological guidelines. In all animals that received subcapsular injections of RenCa cells, an autopsy was done to determine the cause of death.

Tumor-bearing mice were receiving captopril dissolved in drinking water in doses of 25 or 60 mg/kg/d. Twice weekly tumor growth and mice survival were monitored.

**Flow cytometry analysis of tumor-infiltrating lymphocytes.** Tumor-bearing control and captopril-treated mice inoculated with MethA cells were sacrificed 20 days after inoculation (mean tumor diameter, 1 cm). Tumors were excised, minced, and pooled (eight mice per group), and tumor-infiltrating mononuclear cells were isolated by gradient centrifugation. The single-cell suspension was stained with anti-CD8, anti-CD4, anti-CD40 (HTC), anti-CD25, anti-CD62L (phycocyanin), and anti-B220 (allophycocyanin) monoclonal antibodies (PharMingen/Becton Dickinson, San Diego, CA).

**Immunization protocol.** Captopril-treated and control groups of eight 6- to 12-week-old BALB/c mice were immunized s.c. with 1.5 × 10⁷ plaque-forming units of recombinant AdGFP diluted in 100 µL saline. Captopril-treated and control groups of C57BL/6 mice were immunized s.c. with 1.5 × 10⁷ plaque-forming units of recombinant AdLaCZ diluted in 100 µL saline. Eight days after immunization, splenocytes were harvested and processed for CTL and T-helper assays.

In some experiments, 14 days after AdGFP immunization, mice were boosted i.p. with 1.5 × 10⁸ RenCa cells expressing EGFP and splenocytes were harvested 7 days after second immunization.

For antibody response measurement, 14 days after immunization, blood was collected by retro-orbital puncture for serum preparation.

**Detection of antigen-specific CD8⁺ T cells.** Freshly isolated splenocytes obtained from nonimmunized, AdGFP-immunized nontreated, and captopril-treated mice (2 × 10⁶ cells) were incubated with allotypy-cancer-labeled Pro5 MHC pentamer H-2Kd-HYLSTQSAL (ProImmune) at room temperature for 15 minutes in the dark and washed with PBS. Cells were then incubated with phycocerythrin-conjugated anti-mouse CD8 antibody (PharMingen) at 4°C for additional 20 minutes in the dark. After completing the staining process, cells were washed again and then analyzed immediately using a FACSCan flow cytometer (BD Biosciences, San Diego, CA).

**Intracellular IFN-γ staining of CD8⁺ T cells.** Freshly isolated splenocytes obtained from nonimmunized, AdGFP- or AdLaCZ-immunized nontreated, and captopril-treated mice (10⁶ cells) were stimulated with epoetin peptide (0.2 µg/mL) overnight in 1 mL RPMI with 10% FBS and β-glutamine supplemented with 25 ng/mL human recombinant interleukin (IL)-2 (Rendez MedSystems, Vienna, Austria) and 50 µmol/L 2-mercaptoethanol. On the next day, cells were incubated with 1 µL/mL brefeldin A (GoGiPlug, PharMingen) for 6 hours at 37°C, then washed with PBS, and surface stained with allotypycancer-conjugated anti-mouse CD8 antibody. After washing, cells were fixed at 4°C for 20 minutes with Cytofix/Cytoperm (PharMingen), washed with PermWash (PharMingen), and stained for IFN-γ with phycocerythrin-conjugated rat anti-mouse IFN-γ monoclonal antibody (PharMingen). Background staining and threshold levels between positive and negative populations were determined by staining with a phycocerythrin-conjugated isotype control antibody (rat IgG1, PharMingen). Cells were then analyzed using FACSCan flow cytometer.

**Cytokine release assay.** Splenocytes obtained from nonimmunized, AdLaCZ-immunized nontreated, and captopril-treated mice (10⁶ cells per well) were cultured in 1.5 mL RPMI with 10% FBS and β-glutamine in 24-well plates with or without 2.5 µg/mL recombinant β-galactosidase (Sigma-Aldrich, St. Louis, MO). After 72 hours of stimulation, supernatants were tested for IL-2, IL-4, IL-10, and IFN-γ by standard ELISA or Mouse Th1/Th2 Cytokine Cytometric Bead Array (BD Biosciences).

**T-helper proliferation assay.** Splenocytes obtained from nonimmunized, AdLaCZ-immunized nontreated, and captopril-treated mice (1 × 10⁶ cells per well) were cultured in 200 µL RPMI with 10% FBS and β-glutamine in 96-well plates with or without 2.5 µg/mL relevant recombinant proteins for 3 to 5 days. Subsequently, [³H]thymidine (1 µCi/well) was added for 10 hours. Cells were then harvested and the radioactivity was measured using a scintillation counter.

**Detection of antigen-specific antibodies by ELISA.** Specific anti-β-galactosidase antibodies were measured by ELISA on 96-well plates (Maxisorp, Nunc, Greiner, Germany) coated with recombinant β-galactosidase (2.5 µg/mL in PBS, 100 µL/well) and blocked with 3% bovine serum albumin in PBS. Sera obtained from nonimmunized and AdLaCZ-immunized, control, and captopril-treated mice were added in serial dilutions in PBS, starting with 1:200 dilution. After 1-hour incubation, plates were washed four times with PBS-Tween 20 and then incubated with 1:5,000 dilution of horseradish peroxidase– conjugated goat anti-mouse immunoglobulin (Dako Cytomation, Glostrup, Denmark) for 1 hour. After extensive washing with PBS-Tween 20, substrate (OPD tablets, Sigma-Aldrich) was added for 20 minutes at room temperature. After stopping the reaction with 3 mol/L HCl, plates were read in an automated ELISA microplate reader (Bio-Tek Instruments, Denkendorf, Germany) at 490 nm. Each sample was tested at eight log 2 dilutions set up in duplicate wells. Specific titers were determined as the reciprocal of the highest serum dilution giving absorbance value at least 2-fold higher than background.

**In vitro expansion of CTLs.** Splenocytes obtained from AdGFP-immunized, control, and captopril-treated mice were cocultured...
(6 × 10⁶ cells) with irradiated (8 Gy) RenCa cells expressing GFP (1.5 × 10⁴) in RPMI with 10% FBS and l-glutamine supplemented with 50 μmol/L 2-mercaptoethanol and 25 ng/mL recombinant human IL-2 for 3 days. In some experiments, splenocytes were stimulated with epitopic peptide (0.2 μg/mL) in RPMI with 10% FBS and l-glutamine supplemented with 50 μmol/L 2-mercaptoethanol and 25 ng/mL recombinant human IL-2 for 5 to 7 days.

Statistical analyses. Survival curves were analyzed by log-rank test. For the comparison of tumor take rate between groups, a χ² test was used. Differences between samples in immunologic test were analyzed for significance by Student’s t test (two-tailed) or one-way ANOVA test.

Results
Captopril decreases survival of renal cell cancer–bearing, immunocompetent mice in a dose-dependent manner. To evaluate the effect of captopril on survival of mice with immunogenic tumors, RenCa cells were implanted subcapsularly into left kidney. Fourteen days after initiation of the experiment, mice receiving high-dose captopril (60 mg/kg/d) started to die and all animals in this group died after 63 days (median survival, 56 days). In mice receiving low-dose captopril (25 mg/kg/d), the survival time was longer (median, 63 days). The longest survival was observed in the control group (median, 70 days). A significant difference (P = 0.02) in median survival was observed between the high-dose captopril and control groups (Fig. 1A).

To assess a magnitude of specific CD4+ T-cell responses, we studied proliferative responses after stimulation with the whole β-galactosidase protein. Splenocytes from AdLacZ-immunized mice were cultured in vitro with β-galactosidase for 5 days followed by a pulse with [3H]thymidine. Mice immunized with AdLacZ alone had a weak but measurable antigen-specific proliferative response against β-galactosidase. However, captopril treatment during immunization process led to a significant β-galactosidase proliferative response in vitro. The proliferation
rate of splenocytes from captopril-treated group was 3-fold higher compared with control, AdLacZ-immunized mice ($P < 0.001$; Fig. 3A).

To evaluate the immunomodulatory effect of captopril on CD4$^+$ T cells in qualitative terms, splenocytes from AdLacZ-immunized mice were stimulated in vitro with β-galactosidase for 48 hours. After stimulation, culture supernatant was collected and analyzed for IL-2, IL-4, IL-10, and IFN-γ by ELISA. In both control and captopril-treated mice, in vitro restimulation with β-galactosidase induced a comparable cytokine production in splenocytes. Although there was a slightly higher level of cytokine production in captopril-treated group, the difference seemed to be insignificant. These results indicate that captopril does not modulate considerably Th1/Th2 balance in responding lymphocytes in this particular setting (Fig. 3B).

Effect of captopril on the humoral immune responses. Fifteen days after immunization with recombinant AdLacZ, blood samples from control and captopril-treated animals were analyzed for β-galactosidase-specific antibodies. In the captopril-treated group, there was a 16-fold increase ($P < 0.001$) of anti-β-galactosidase antibodies compared with the control group (Fig. 3C).

Captopril impairs development of antigen-specific CD8$^+$ T cells. To evaluate the effect of captopril on mounting of antigen-specific CD8$^+$ T-cell responses, two model antigens (GFP and β-galactosidase) were used.

In the first set of experiments, control and captopril-treated mice were immunized with the recombinant adenoviral vector encoding GFP. The population of GFP-specific, CD8$^+$ splenocytes of captopril-treated animals 14 days after immunization followed by 7 days of in vitro restimulation was ∼2-fold smaller than of control mice (Fig. 4A).

In the next set of experiments, to evaluate the effect of captopril on formation of antigen-specific, IFN-γ-secreting CD8$^+$ T cells, another model antigen (β-galactosidase) was used in C57BL/6 mice. As shown on Fig. 4B, AdLacZ immunization induced weak but measurable antigen-specific CTL responses. However, in contrast to control mice, where ∼0.6% of circulating CD8$^+$ T cells displayed the β-galactosidase specificity, captopril-treated animals did not mount any antigen-specific CTL response (Fig. 4B).

Captopril increases infiltration of immunogenic tumors by B cells. Immunophenotypic analysis of tumor-infiltrating lymphocytes was carried out to shed more light on the possible mechanisms involved in promotion of tumor growth in captopril-treated animals. Flow cytometry analysis of tumor-infiltrating lymphocytes did not reveal significant differences in the T-cell infiltrate from each group studied (Fig. 5A-C). However, the MethA tumors in the captopril-treated animals were more densely (+30%) infiltrated by B cells as shown by anti-CD40 and anti-B220 staining (Fig. 5D).

Discussion

There are five major findings of these studies. Captopril (a) promotes growth of immunogenic tumors, (b) promotes tumor recurrence in minimal residual disease, (c) impairs generation of antigen-specific CD8$^+$ T cells, (d) enhances infiltration of immunogenic tumors by B cells, and (e) increases production of antigen-specific antibody.

We employed an orthotopic animal model of renal cell cancer, which, in contrast to the s.c. model, resembles clinical course of renal cancer in human in terms of growth pattern, metastatic potential, and responsiveness to systemic treatment (27). Other orthotopic tumor models, such as a prostate cancer model, have also shown importance and specificity of a microenvironment on tumor biology (28). In various in vivo studies, a broad range of captopril doses have been evaluated (19-94 mg/kg/d; refs. 13, 29, 30). We have concentrated on the
effect of captopril on tumor growth at doses that were well tolerated by animals in our study.

In studies of Small et al. and Hii et al., high doses of captopril (80 and 94 mg/kg/d, respectively) markedly reduced tumor growth and its metastatic potential and extended survival of treated mice. However, Small et al. used a low immunogenic mammary carcinoma cells (MA-16), whereas Hii et al. employed a xenogenic model of human renal cell cancer cells implanted into immunocompromised scid/scid mice (29, 30). Moreover, low doses of captopril (20 mg/kg/d) were also shown to inhibit growth of particular tumors (e.g., murine hepatoma; ref. 14). In contrast, in our studies, captopril promoted growth of renal cell cancer in an orthotopic model in mice. Moreover, adjuvant administration of captopril to mice with resected renal tumors shortened their survival, which indicated that captopril might also enhance progression of a minimal residual disease. The 25% survival rate of nontreated animals after resection of primary kidney tumor resembles clinical conditions, where 10-year survival rate of localized renal cancer patients who underwent nephrectomy varies between 20% and 50% (31). Our studies employing immunocompetent and immunocompromised mice and high and low immunogenic tumors (MethA and B78-H1 cells, respectively) clearly showed that the tumor-promoting properties of captopril were associated with its influence on the immune system. We have not observed such spectacular antitumor effects of captopril in scid/scid mice as described by Hii et al. most likely due to a significantly lower dose of captopril used. The antitumor effect of captopril in the above-mentioned studies was only observed in mice receiving the highest dose of

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**Fig. 3.** Effect of captopril on particular variables of immune response. A, T-helper immune responses in vivo are increased by captopril. Splenocytes from AdLacZ-immunized control or captopril-treated mice were stimulated in vitro with β-galactosidase in 5 days [3H]thymidine proliferation assay. Splenocytes from mock-immunized mice were used as control. Captopril is able to enhance T-helper immune responses induced by AdLacZ vaccine by a factor of 2 to 3. *, P < 0.001. B, captopril does not modify Th1/Th2 balance. Splenocytes from AdLacZ-immunized, control, and captopril-treated mice were stimulated with purified β-galactosidase. After 48 hours of culture, supernatant was harvested and assayed for cytokine content by ELISA. Splenocytes from naive mice were used as control. C, captopril increases production of β-galactosidase-specific antibodies in AdLacZ-immunized mice. Groups of mice were immunized with AdLacZ and fed with captopril or drinking water or plain water. After 2 weeks, serum was collected and tested on ELISA plates coated with β-galactosidase. Data are endpoint titers defined as the inverse of the highest dilution that resulted in an absorbance value twice greater than that of the preimmune serum with a cutoff value of 0.05. *, P < 0.001.
the drug (94 mg/kg/d). In our experiments, similar doses of captopril were toxic and caused a significant mortality of treated animals (data not shown). However, the slightly delayed development of nonimmunogenic B78-H1 murine melanoma tumors in captopril-treated, immunocompetent mice may confirm the existence of antitumor potential of captopril in particular conditions.

The behavior of immunogenic tumors suggests that captopril may promote tumor growth through regulation of the immune system. Captopril is known for its immunomodulatory properties (32). It inhibits T-cell reactivity (33, 34), suppresses IL-12 production (35), induces immune unresponsiveness, and prolongs allograft survival (36). Results of our studies indicated that the tumor-promoting activity of captopril depended on a modulation of host immune system. First, indirect evidence came from observations of highly immunogenic tumors, such as MethA and low immunogenic B78-H1 melanoma tumors. Enhanced formation of tumors in all captopril-treated animals after injection of suboptimal dose of MethA cells strongly indicated its tumor-promoting activity. However, captopril did not affect MethA tumor growth in immunodeficient mice. Thus, in an organism, which is unable to mount an immune response against tumor antigens due to either a host immune incompetence or low tumor immunogenicity, captopril is not showing its tumor-promoting properties and may display antitumor activity associated with inhibition of tumor vessel formation.

Assuming that captopril may influence the immune system, which in turn controls growth of immunogenic tumors, we evaluated immunomodulatory functions of captopril in vivo. Because precise quantitative analyses of immune responses to undefined tumor antigen are problematic, we decided to use GFP and β-galactosidase as model antigens. Therefore, to assess the immunomodulating activity of captopril in vivo, we immunized groups of control and captopril-treated mice with an adenoviral vector encoding either β-galactosidase or GFP. Immunization of control mice with an AdGFP vector generated ~10% of antigen-specific CD8+ T cells. However, we showed that treatment of immunized mice with captopril decreased the GFP-specific population of CD8+ T cells by 50%. By using another model antigen (β-galactosidase), we confirmed the observed inhibitory effect of captopril on generation of functional, antigen-specific CTLs. In nontreated animals, AdLacZ immunization induced measurable T-cell-mediated responses against adenovirus-derived β-galactosidase. The relatively low frequency of β-galactosidase-specific, IFN-γ-secreting CD8+ T cells generated by immunization of mice with AdLacZ (0.6%) was similar to frequencies of specific CTLs observed in human after immunization with vaccinia or variola viruses (37). Moreover, we observed that administration of captopril significantly augmented infiltration of immunogenic tumors by activated B cells and boosted specific, anti-β-galactosidase antibody response. Such a behavior could indicate a shift of the ongoing immune reaction toward Th2-type response. However, to our surprise, we did not observe any changes in cytokine profiles, which would indicate an Th2 shift.

Tumors employed in our studies did not express MHC II molecules. Accordingly, induction of tumor immunity depended exclusively on priming of CD4+ T cells by MHC II+ antigen-presenting cells and then activation of CTL by MHC class I–restricted antigens (38, 39). It is possible that the mechanism of inhibition of T-cell-dependent immunity by captopril was associated with an increased number and activity of tumor-infiltrating B cells (40). It is acknowledged that B cells can compete with dendritic cells for tumor antigens. Although
B cells can also present antigens to T cells, they are far less efficient than dendritic cells (41). It is known that B cells are very inefficient in cross-priming and thus are not able to stimulate T cells against tumor antigens (42). The MHC product and MHC-peptide complexes are 10 to 100 times lower on B cells than on dendritic cells (43). Moreover, captopril was shown to directly impair immunostimulatory functions of dendritic cells by inhibition of their endogenous renin-angiotensin system (44). An increased proliferation of antigen-specific CD4+ T cells from captopril-treated mice does not exclude the possibility that some functions of these cells were impaired by captopril during the induction phase of immune response, which could not be detected by in vitro assays. Qin et al. have clearly showed that B cells can impair functions of

![Image](image_url)

**Fig. 5.** Flow cytometry analysis of lymphocytes infiltrating s.c. MethA tumors in control and captopril-treated mice. There were no significant differences between both control and captopril-treated mice in CD4+CD25+ T cells (A), CD4+CD62L T cells (B), and CD8+CD62L T cells (C). However, in the captopril-treated mice, MethA tumors contained a significantly larger population of active CD40+B220+ B cells (30%) compared with control animals (D).
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