

With respect to the local RAS in the prostate gland, Ang-II has been reported to be present in human seminal fluid at a concentration three- to fivefold higher than that found in blood [5]. An *in vitro* study revealed that Ang-II affected sperm function through the AT₁ receptor to increase oocyte-penetrating ability [6,7]. Furthermore, there is assembled evidence that the male reproductive organs contain RAS components, such as angiotensinogen, renin, angiotensin I converting enzyme, and AT₁ and AT₂ receptors. The accumulated evidence and our previous results suggest that the prostate itself seems to be a target for angiotensin, and the prostatic RAS may affect the development of prostate cancer.

Aminopeptidase A and N (APA and APN) have been identified as convertases of angiotensins; Ang-II to angiotensin III (Ang-III), and Ang-III to angiotensin IV (Ang-IV), respectively (Fig. 1). On the other hand, they are also considered to be cell-surface peptidases in the form of integral membrane proteins, which are involved in the control of cell proliferation and differentiation by modulating the access of peptides to their membrane receptors [8–10]. Alterations of peptide activities by abnormal expression or catalytic functions of cell-surface peptidases are likely to contribute to neoplastic transformation or progression.

Generally, cancer development and progression are caused by many factors that are involved in DNA mutation and abnormal activity of oncogenes or tumor suppressor genes. Activation of growth factor receptors and many growth factors also contributes to cancer development and progression. Prostate cancer has unique features in that androgens and the androgen receptor (AR) play an important role in its development and progression as well as in the normal prostate and in benign prostatic hypertrophy (BPH) progression [11].

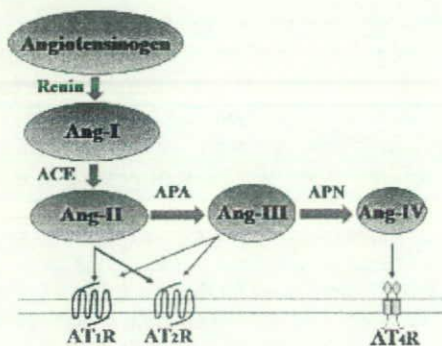


Fig. 1. Angiotensin cascade and angiotensin receptors. Aminopeptidase A (APA) converts Ang-II to Ang-III, and aminopeptidase N (APN) converts Ang-III to Ang-IV in the renin-angiotensin system (RAS). Ang-II and Ang-III bind to the AT₁ and AT₂ receptors, and Ang-IV binds to the AT₄ receptor.

Furthermore, prostatic growth and differentiation are proposed to be regulated by autocrine or paracrine factors secreted by prostatic epithelial and stromal cells [12]. Prostate cancer is initially hormone dependent, and growth and protection against apoptosis are controlled by androgen; however, hormone-dependent prostate cancer later becomes hormone independent. Although it is still unclear how prostate cancer growth changes from being hormone dependent to hormone independent, AR activation such as amplification, mutation and related AR signaling is considered to play a key role [13–15].

The present study was carried out to obtain a deeper insight into RAS regulation in prostate cancer. We focused on the biological role of Ang-III and the expression of Ang-III-related convertases in prostate cancer cells and tissue. We investigated the effects of Ang-III on proliferation in prostate cancer cells, and, furthermore, the expression of APA and APN in prostate specimens from patients with prostate cancer. Our results support the hypothesis that a tissue RAS may be activated in prostate cancer and be influenced by several kinds of hormonal stimulation in prostate cancer cells.

MATERIALS AND METHODS

Cell Lines

DU145 and LNCaP cells, human prostate cancer cell lines, were obtained from the American Type Culture Collection (Rockville, MD). DU145 cells were cultured in MEM medium and LNCaP cells in F-12 medium, and both cells were supplemented with 10% fetal calf serum (FCS) under 5% CO₂ before the experiments. In the experiments, these cells were cultured in phenol red-free RPMI plus 0.1% bovine serum albumin (BSA) or F-12 medium with charcoal-stripped 10% FCS, and stimulated with reagents.

Reagents

Ang-III was purchased from Sigma (Atlanta, GA). Anti-phospho-MAP kinase antibody and anti-MAP kinase antibody were purchased from Cell Signaling Technology (Beverly, MA). Bicalutamide was provided by AstraZeneca (London, UK) and olmesartan by Dai-ichi Sankyo Pharmaceutical Co. (Tokyo, Japan).

Cell Growth Analysis

Cell growth was estimated by counting the cell number using a MICROCELLCOUNTER (Toha Co., Tokyo). Briefly, LNCaP and DU145 cells were seeded onto 24-well plates at a density of $2-5 \times 10^4$ cells/well.

Cells were treated with Ang-III at various concentrations as indicated in the figures for 5 days. Simultaneously, the cells were pretreated with olmesartan for 30 min, and cultured in phenol red-free RPMI plus 0.1% BSA in the presence of Ang-III for 5 days. After incubation in 5% CO₂ at 37°C, cells were harvested with trypsin and cell numbers were determined with a cell counter on day 5.

Patients and Tissue Specimens

The study group consisted of 47 patients with prostate cancer at Yokohama City University Hospital; 39 with previously untreated prostate cancer who had undergone radical prostatectomy and 8 with recurrent HRPC after maximal androgen blockade therapy with an anti-androgen agent plus LHRH analogue. All samples were flash-frozen in liquid nitrogen as soon as they were obtained at operation, and immediately within 30 min stored at -80°C until analysis. In untreated prostate cancer, trimmed prostate blocks obtained by radical prostatectomy were cut into 10- μ m-thick sections in a cryostat. After mapping of carcinoma foci revealed in paraffin sections stained with hematoxylin and eosin, the carcinoma region and normal tissue region were dissected under a three-dimensional microscope (Olympus, Tokyo). In HRPC, samples were obtained from lymph node metastases in three cases, bone metastases in two cases, prostate in two cases and liver metastases in one case. Informed consent was obtained from all of the patients and families in this study.

Real-Time Quantitative PCR

As previously reported, we performed real-time quantitative PCR on a total of 47 prostate specimens. Total RNA was extracted with ISOGEN. To quantify expression of RAS components, real-time quantitative PCR analysis was performed with an iCycler (Bio-Rad Inc.). The sequences of oligonucleotides as forward or reverse primers of APA, APN and β -actin were as follows:

APA—forward: 5'-TTC CTC CGT GTT AGG GTT TGC-3', reverse: 5'-ATA CCG ATA CAC CAG AAG CCT GAG-3'; APN—forward: 5'-AGC CCA CCT GGA ACT TGA AATTC-3', reverse: 5'-AGA TGG CGT CAA ACA GCT CA-3'; β -actin—forward: 5'-TAA TAC GAC TCA CTA TAG GGA GAG CGG GAA ATC GTG CGT GAC ATT-3', reverse: 5'-GAT GGA GTT GAA GGT AGT TTC GTG-3'.

β -Actin was used as an internal control. Sequence-specific primers were designed using computer soft-

ware, Primer Express (Applied Biosystem, Inc.). Reagents in iQ SYBR Green Supermix (Bio-Rad, Inc.) were used according to the manufacturer's protocol. PCR reaction was performed in a total volume of 25 μ l including 7.5 pmol of each primer and 1.0 μ l cDNA synthesized with random hexamers. PCR conditions were 3 min at 95°C for pre-heating, then 30 sec at 95°C, 30 sec at 57°C, and 1 min at 72°C for 40 cycles. All specific quantities were divided by the quantity of β -actin.

Western Blot Analysis

LNCaP and DU145 cells were cultured in phenol red-free RPMI plus 0.1% BSA for 24 hr before the experiments. Then, cells were harvested after Ang-III treatment as indicated in the figures. Cells were pretreated with olmesartan for 30 min, and then stimulated with Ang-III, and harvested at 15 min for investigation of the phosphorylation of MAPK. DU145 and LNCaP cells were cultured in phenol red-free RPMI plus 0.1% BSA, and harvested after incubation with Ang-III and/or olmesartan for 24 hr at the indicated concentrations in the figures. LNCaP cells were also cultured in phenol red-free RPMI plus 0.1% BSA, and harvested after incubation with dihydrotestosterone (DHT), estradiol (E2), dexamethasone (Dex), or bicalutamide (Bcl) for 24 hr. Cells in the appropriate conditions were washed twice with ice-cold phosphate-buffered saline (PBS), lysed in ice-cold buffer consisting of 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% Nonidet P-40, 100 mM sodium fluoride, 200 mM sodium orthovanadate, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin and 3 mg/ml aprotinin, and centrifuged (30 min, 4°C, 14,500g). Following quantitation, 20 or 40 μ g of each cell lysate was added to SDS gel-loading buffer (containing a reducing agent) and boiled for 5 minutes. The samples were subjected to SDS-PAGE on 10% gel, and electrotransferred to Immobilon-P (Millipore, Bedford, MA). After blocking the membrane with 5% albumin, Western blotting was performed using the antibody of interest, and the product was detected with an ECL detection system (Amersham Int.). Data of densitometry were calculated as the mean relative density of bands compared with control samples. The results were presented as means \pm standard deviation (SD) (n = 3).

Statistical Analysis

Significance was examined by ANOVA followed by *t*-test, and differences with *P* < 0.05 (*) were considered significant. Mann-Whitney *U*-test was used to analyze the significance of expression of APA and APN.

RESULTS

To investigate the effect of Ang-III on human prostate cancer cells, we applied it in LNCaP (androgen-dependent) and DU145 (androgen-independent) cells. As shown in Figure 2A,B, Ang-III treatment increased both prostate cancer cells in a dose-dependent manner. Because earlier reports indicated that Ang-III can bind to the AT₁ receptor, which is predominantly for Ang-II (Fig. 1) [16], we investigated the effect of an AT₁ receptor blocker on cell proliferation induced by Ang-III. Olmesartan is a selective blocker of the AT₁ receptor and is widely used as anti-hypertensive agent. As shown in Figure 2A,B, olmesartan significantly suppressed the cell growth induced by Ang-III treatment in prostate cancer cells.

Next, in order to confirm stimulation of cell proliferation via signal transduction activation by Ang-III treatment, we carried out Western blot of the phosphorylation of MAPK activated by Ang-III. As anticipated, phosphorylation of MAPK was activated in LNCaP and DU145 cells (Fig. 3A,B). Simultaneously, the phosphorylation was inhibited when cells were treated with olmesartan.

We then performed real-time quantitative PCR using human prostate cancer tissue. PCR data showed that APA and APN were identified in all 86 prostate tissue samples (39 normal prostate tissue, 39 untreated prostate cancer tissue, and 8 recurrent cancer tissue). There were no significant differences in APA mRNA expression between normal, untreated cancer and hormone-refractory prostate cancer (HRPC) tissue (Fig. 4A). On the other hand, APN mRNA expression in HRPC tissue was significantly lower than that in normal prostate tissue and untreated prostate tissue ($P < 0.01$) (Fig. 4B). From the viewpoint of pathological grade and stage, APA and APN expression showed no

significant difference between normal, untreated cancer and HRPC tissue (data not shown). With regard to clinical features, we investigated the correlation between APA and APN expression and outcome in eight HRPC cases in which clinical information could be compiled. However, there was no significant correlation between their expression and recurrence-free survival or overall survival period in HRPC patients (data not shown).

We examined the protein expression of APA and APN when LNCaP or DU145 cells were cultured under Ang-III stimulation for 24 hr. Figure 5A shows that APN expression of LNCaP cells was augmented by Ang-III stimulation in a dose-dependent manner. Similarly, APN expression of DU145 cells was augmented by Ang-III stimulation as shown in Figure 5B. On the other hand, although LNCaP cells expressed APA protein, it was not regulated by Ang-III stimulation or treatment with an ARB, olmesartan, for 24 hr (Fig. 5C). APA expression in DU145 cells was not influenced by Ang-III stimulation or olmesartan (data not shown).

LNCaP cells are androgen-dependent prostate cancer cells because they have androgen receptors. LNCaP cells were stimulated with androgen, DHT, or various agents such as E2, Dex or Bcl, which are used in practice for prostate cancer, for 24 hr, and we examined the expression of APA and APN by Western blot. Figure 6 shows that each agent enhanced APA expression in LNCaP cells. In particular, E2 augmented its expression compared to control (no treatment), while APN expression was not regulated by either agent.

DISCUSSION

In a previous study, real-time RT-PCR revealed that RAS components were expressed in prostate cancer

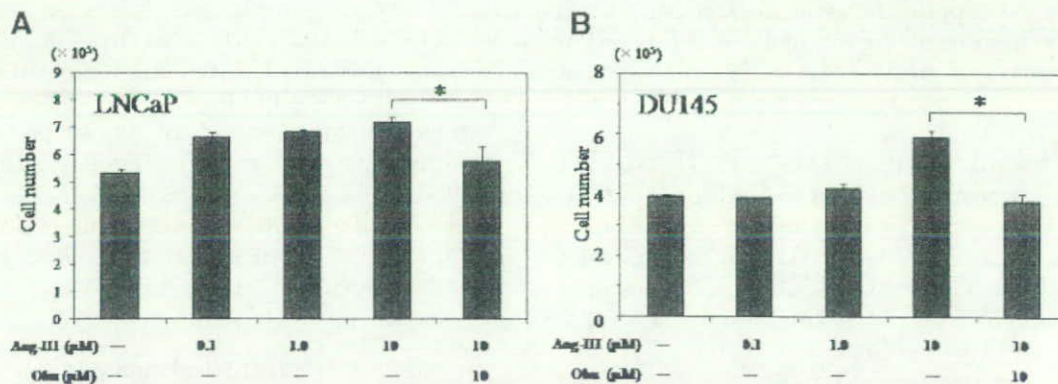


Fig. 2. Cell growth of established prostate cancer cells stimulated with angiotensin III (Ang-III). **A:** Cell number of LNCaP stimulated with Ang-III for 5 days increased in a dose-dependent manner. Cell proliferation induced by 10 μM Ang-III was inhibited by 10 μM olmesartan treatment. **B:** Cell number of DU145 stimulated with Ang-III for 5 days increased in a dose-dependent manner. Cell proliferation induced by 10 μM Ang-III was inhibited by 10 μM olmesartan treatment. Bars represent mean ± standard deviation (SD). * $P < 0.01$, $n = 4$.

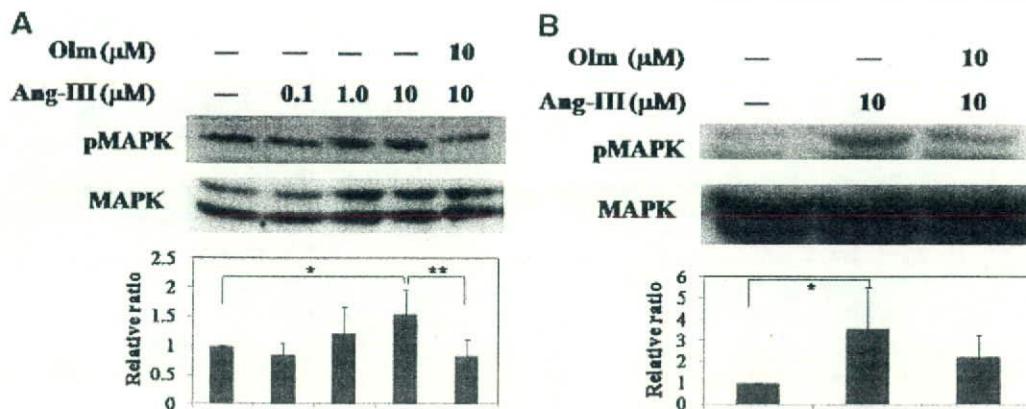


Fig. 3. Western blots of phosphorylated MAPK in prostate cancer cells stimulated with Ang-III. LNCaP and DU145 cells were harvested after 15 min of stimulation with angiotensin III and olmesartan. The cells were lysed, and detergent extracts were immunoblotted with anti-phospho-MAPK or MAPK antibodies. **A:** In LNCaP cells stimulated dose-dependently with Ang-III, the phosphorylation of MAPK was activated, and it was inhibited by olmesartan treatment. **B:** In DU145 cells stimulated with Ang-III, the phosphorylation of MAPK was activated, and it was inhibited by olmesartan treatment. In **A** and **B**, densitometries of the developed blots were performed, and the ratios between the intensity of pMAPK and control MAPK were determined. A: * $P < 0.05$, ** $P < 0.04$, $n = 3$. B: * $P < 0.05$, $n = 3$.

cells, irrespective of their possessing the androgen receptor, and were expressed more highly in HRPC tissue than in normal and untreated prostate cancer tissue [17]. These data indicated an important role of Ang-II in the development of hormone refractory prostate cancer. As the next step, in the present study, we focused on the biological role of Ang-III, which is converted from Ang-II, and its converting enzymes, APA and APN. Similarly to Ang-II, Ang-III activated the cell growth of prostate cancer, and interestingly, it was inhibited by olmesartan, an ARB. The proliferative signal transduction of Ang-III also seems to be via MAPK phosphorylation, as for Ang-II [4]. Most noteworthy is that real-time PCR showed that APN mRNA expression in HRPC tissue was significantly lower than that in normal prostate tissue and untreated prostate

tissue. The lower level of APN expression in HRPC tissue leads to the accumulation of Ang-III, which supports the hypothesis that Ang-III may be involved at least in part in HRPC development. More interestingly, some agents, such as E2 and Dex, that are usually used for HRPC induced APA expression in LNCaP cells, whereas they did not regulate APN expression.

Recently, the role of the local RAS has been shown to be noteworthy, especially in the development of various cancers [18]. The RAS has classically been identified in reno-cardiovascular organs including the kidney, heart and vessel walls, where its enzymatic actions and produced peptides are released systemically, leading to blood pressure regulation and electrolyte/fluid homeostasis. Among the RAS components, Ang-II exerts biologic activity by binding to two

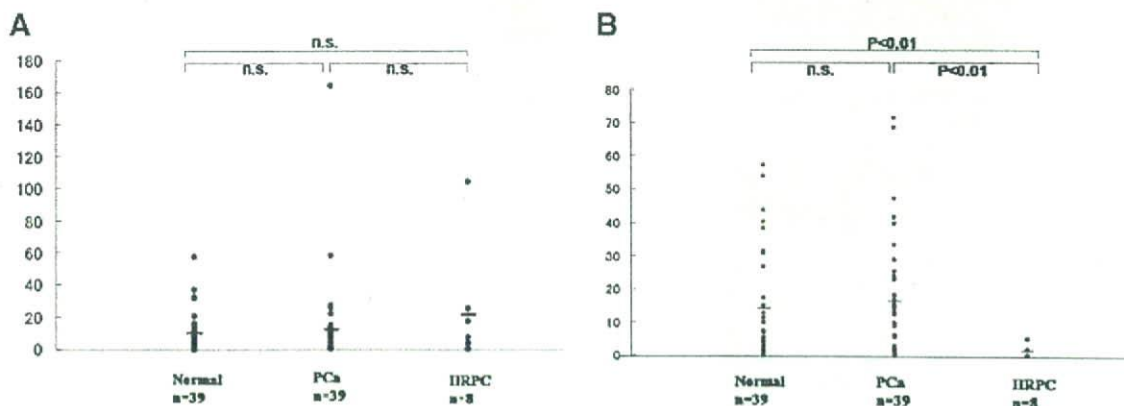


Fig. 4. mRNA expression of APA (**A**) and APN (**B**) in prostate tissue. Real-time quantitative PCR was performed to compare their mRNA expression in prostate tissue (normal, untreated primary prostate cancer and HRPC). Each mRNA expression level was divided by β -actin expression level for normalization. **A:** There was no significant difference between the three groups. **B:** Expression of APN was significantly lower in HRPC tissue than in normal or untreated primary prostate cancer tissue (Mann-Whitney's U -test, $P < 0.01$).

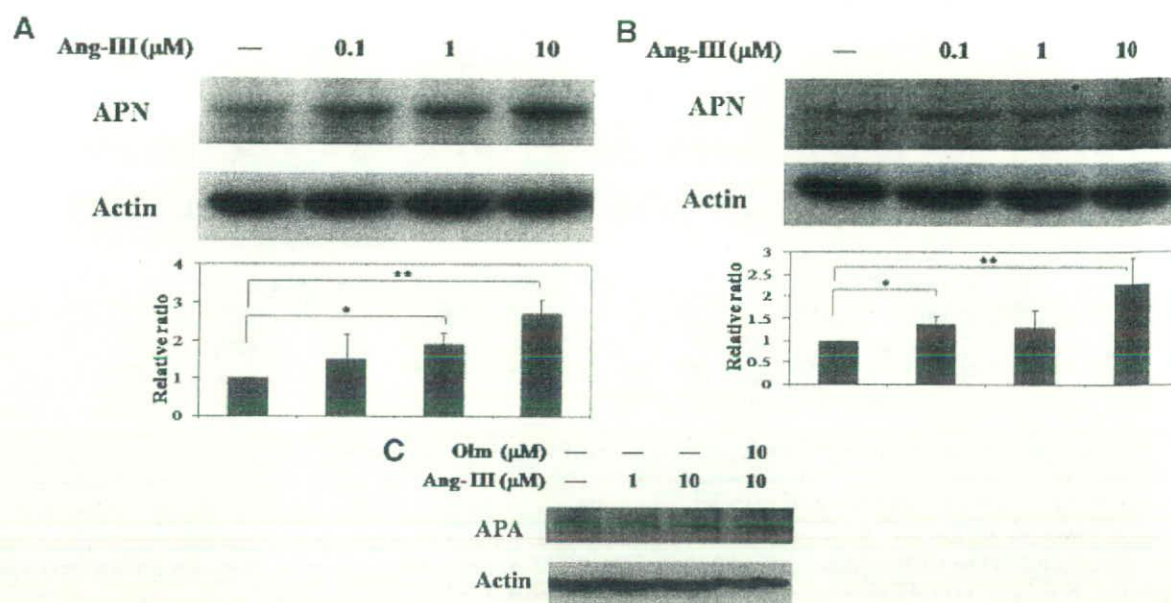


Fig. 5. Western blots of APN and β -actin in LNCaP (**A**) and DU145 cells (**B**). Cells were harvested after 24 hr of stimulation with Ang-III. They were lysed, and detergent extracts were immunoblotted with anti-APN or anti- β -actin antibodies. In both cells, APN protein expression was enhanced by Ang-III stimulation in a dose-dependent manner. Densitometries of the developed blots were performed, and the ratios between the density of APN and control β -actin were determined. * $P < 0.01$, $n = 3$. **C:** Western blots of APA and β -actin in LNCaP cells. Cells were harvested after 24 hr of stimulation with Ang-III with or without olmesartan. They were lysed, and detergent extracts were immunoblotted with anti-APA or anti- β -actin antibodies. APA protein expression was not regulated by Ang-III or olmesartan treatment.

types of receptors; AT_1 and AT_2 [19,20], which are functionally distinct polypeptides with 30% sequence homology and with seven transmembrane domains, belonging to the guanosine phosphate binding protein coupled receptor (GPCR) family. We previously reported that the AT_1 receptor binds to Ang-II with

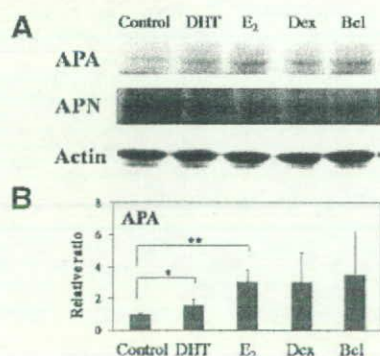


Fig. 6. Western blots of APA, APN, and β -actin in LNCaP cells. **A:** Cells were harvested after 24 hr of stimulation with DHT, estradiol (E₂), dexamethasone (Dex) or bicalutamide (Bcl). They were lysed, and detergent extracts were immunoblotted with anti-APA, anti-APN or anti- β -actin antibodies. APA protein expression was enhanced by treatment with each agent. **B:** Densitometry of the developed blots was performed, and the ratios between the density of APA and control β -actin were determined. * $P < 0.04$, ** $P < 0.01$, $n = 3$.

concentration-dependent affinity [4,21]. Likewise, Ang-III appears to bind to the AT_1 receptor in a concentration-dependent manner, resulting in cell proliferation, as shown in this study. This speculation is supported by the data that olmesartan inhibited cell growth of prostate cancer induced by Ang-III as shown in Figure 1. Ang-II and Ang-III have been traditionally believed to bind to the AT_1 and AT_2 receptors [16] (Fig. 1). Interestingly, Ang-III was shown to be several times more effective in the brain than Ang-II [22]. In prostate cancer, there have been few reports besides our previous report in which AT_1 receptor expression was shown to be higher in cancer tissue than in normal prostate tissue by RT-PCR analysis [4]. Further comparative investigations of AT_1 receptor expression have confirmed higher expression in HRPC tissue [17]. Accordingly, based on the data of an increase in the AT_1 receptor and accumulation of Ang-III in HRPC tissue, it is hypothesized that Ang-III may also play a pivotal role in the development of HRPC.

Although real-time PCR analysis in the present study showed no significant difference in APA expression between normal, untreated cancer and HRPC tissue (Fig. 4A), western blotting data (Fig. 6) implied the potential of APA in the development of HRPC. In brief, E₂ and Dex, which are used for hormone-independent cancer, were able to induce APA

expression in prostate cancer cells, which has similarly been reported by Katsumata et al. [23] who demonstrated that APA expression was stimulated by progesterone in human choriocarcinoma cells. A recent report indicated that APA was up-regulated and enzymatically active in blood vessels of human tumors, but was not detected in normal blood vessels [24]. Given that APA mainly plays a role as a converting enzyme from Ang-II to Ang-III, it is possible that APA is implicated in neovascularization of tumor development. In addition to being present in blood vessels, APA expression was found on dysplastic cells and was increased in precancerous lesions and invasive cervical cancer [25]. These data, including our own, suggest that APA may play a regulatory role in neoplastic transformation and disease progression in various cancers.

Several kinds of carcinomas including those of colon, breast and lung were reported to show little expression of APN [26,27], which coincides with the data of prostate cancer reported by Bogenrieder et al. [28] demonstrating that APN expression (immunoreactivity) was minimal or absent in 79% of primary prostate cancer specimens and in 64% of metastatic tumor specimens. The present data of RT-PCR analysis (Fig. 4B) were similar to those data. Some reports also demonstrated that the expression of APN in renal cancer tissue was lower than that in adjacent normal tissue [29,30]. Although APN has previously been considered as a proteolytic enzyme with ability to facilitate tumor cells invade through the extracellular matrix [31,32], lower expression of APN in HRPC implies that APN may enzymatically function in different manners from extracellular matrix degradation. Consequently, it should be noted that APN functions as a convertase of Ang-III to Ang-IV. We have previously demonstrated that a local prostatic RAS is involved in the development of prostate cancer and HRPC. In accordance with the angiotensin converting cascade, our findings of no change in APA and a decrease in APN expression suggest that the metabolism of Ang-II to Ang-III is constant, whereas the metabolism of Ang-III to Ang-IV is slow, resulting in the predominant actions of Ang-II and Ang-III in prostate cancer cells. We hypothesize that these angiotensins may synergistically stimulate cell growth and angiogenesis.

In conclusion, although the biological role of aminopeptidases such as APA and APN in cancer cells remains unexplained, it is noteworthy to explore them with respect to their actions as convertases in the local RAS in cancer tissue. The current study documented the biological action of Ang-III in prostate cancer cells and decreased expression of APN in HRPC compared to normal or untreated prostate cancer tissue. We interpret these phenomena to reflect the involvement of

the prostatic RAS in the development of prostate cancer and hormone refractory status. Further exploration to elucidate the precise biological effects of Ang-III and aminopeptidases is anticipated to lead to the development of anti-cancer drugs against HRPC.

ACKNOWLEDGMENTS

The original studies in our program were supported by a grant for the 2007 Suzuki Foundation, Yamaguchi Endocrine Disease Foundation, a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Science and Technology, the Third-term Cancer Control Strategy Program from the Ministry of Health, Labour and Welfare, and 2008 Strategic Research Projects (No. K20003 and K20010) of Yokohama City University, Japan.

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Review Article

Pharmacology and new perspectives of angiotensin II receptor blocker in prostate cancer treatment

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Abstract: Although a low prevalence of cancer in hypertensive patients receiving angiotensin converting enzyme inhibitors has been reported, the molecular mechanisms have not been elucidated. It is known that angiotensin-II (Ang-II) plays a fundamental role not only as a vasoconstrictor in controlling blood pressure and electrolyte and fluid homeostasis, but also as a mitogenic factor through the Ang-II type-1 (AT1) receptor in cardiovascular cells. Interestingly, there is increasing evidence that the renin-angiotensin system (RAS) is implicated in the development of various cancers. As we previously reported, AT1 receptor blockers (ARBs), a class of antihypertensive agent, have the potential to inhibit the growth of prostate cancer cells and tumors through the AT1 receptor. This review provides an insight into the key role of Ang-II and the AT1 receptor, and the possibility of ARBs for molecular targeting of mitogenesis and angiogenesis in prostate cancer.

Key words: angiotensin II, angiotensin II receptor, angiotensin II receptor blocker, prostate cancer, renin-angiotensin system.

Introduction

Prostate cancer is the most common malignant disease in men and a frequent cause of cancer death in the United States¹ and Western countries. Recently, the prevalence of prostate cancer has also been increasing in Japan.² Although the etiology is not clear, one cause is considered to be westernization of the diet and lifestyle in Japan, and another is thought to be altered gene expression in prostate cells by several environmental factors.³

Since Huggins and Hodges first reported hormonal therapy (androgen ablation therapy) for prostate cancer, such therapy with the concept of androgen ablation or blockade of androgen's action has remained critical and universal, especially for advanced prostate cancer.⁴ Despite hormonal therapy having good efficacy for patients with advanced prostate cancer initially, most patients develop resistance to treatment (hormone-refractory cancer) within several years, and their survival remains poor. Many growth factors and cytokines are thought to be involved in the development of hormone-refractory prostate cancer (HRPC).

Interestingly, it has been reported that angiotensin II (Ang-II) is implicated in the development or invasion of several kinds of cancer tissue, including breast, ovarian and pancreatic cancer.⁵ There is clinical evidence supporting the involvement of Ang-II in carcinogenesis. Lever *et al.* carried out a retrospective cohort study that raised the possibility of protection against cancer by the use of angiotensin converting enzyme (ACE) inhibitors.⁶ Following that report, several studies concerning the association between antihypertensive medication and cancer risk have been reported. Regarding prostate cancer, Ronquist *et al.* found that users of captopril, an ACE inhibitor, showed a lower risk of subsequent prostate cancer, based on data from the General Practice Research Database in the UK.⁷

As we previously reported that Ang-II is a growth factor, an Ang-II receptor blocker (ARB) could inhibit the proliferation of prostate cancer,⁸ especially in hormone-refractory cancer. To confirm the role of Ang-II in cancer cell proliferation and migration, several experiments have been carried out, suggesting that this peptide might also be involved

in cancer development. This review highlights that renin-angiotensin system (RAS) plays a potential role in various aspects of prostate cancer, and ARBs could be useful for prostate cancer or its chemoprevention.

Role of androgen receptor in development and progression of prostate cancer

Generally, cancer development and progression are caused by many factors that are involved in DNA mutation and abnormal activity of oncogenes or tumor suppressor genes. Activation of growth factor receptors and many growth factors also contributes to cancer development and progression. Similarly to other cancers, these factors are involved in the development and progression of prostate cancer. In addition to these factors, prostate cancer has unique features in that androgens and a cognate receptor, the androgen receptor (AR), play an important role in its development and progression (including in the normal prostate and in benign prostatic hypertrophy [BPH] progression).⁹ In addition, prostate cancer is initially hormone dependent, and growth and protection against apoptosis are controlled by androgen; however, hormone-dependent prostate cancer later becomes hormone independent. Although it is still unclear how prostate cancer growth changes from being hormone-dependent to hormone-independent, AR activation such as amplification, mutation and related AR signaling is considered to play a key role (Fig. 1).^{10–12}

As other factors related to AR activation, AR cofactors are considered to be important for AR transactivation (Fig. 1). Some AR cofactors are known to be up- or downregulators in prostate cancer. Deregulation of the expression of some AR co-factors or the interaction between AR and AR cofactors has been shown in many studies.¹³ For example, ARA55 expression in HRPC is lower than that in BPH and untreated prostate cancer. Moreover, higher ARA55 expression was associated with shorter recurrence-free survival and overall survival in hormone-refractory prostate cancer patients.¹⁴

Growth factors and cytokines associated with the development of hormone-refractory prostate cancer

In addition to the abnormal function of AR, many growth factors and cytokines secreted from surrounding cells have also been identified and

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Received 25 August 2007; accepted 20 September 2007.

Online publication 19 November 2007

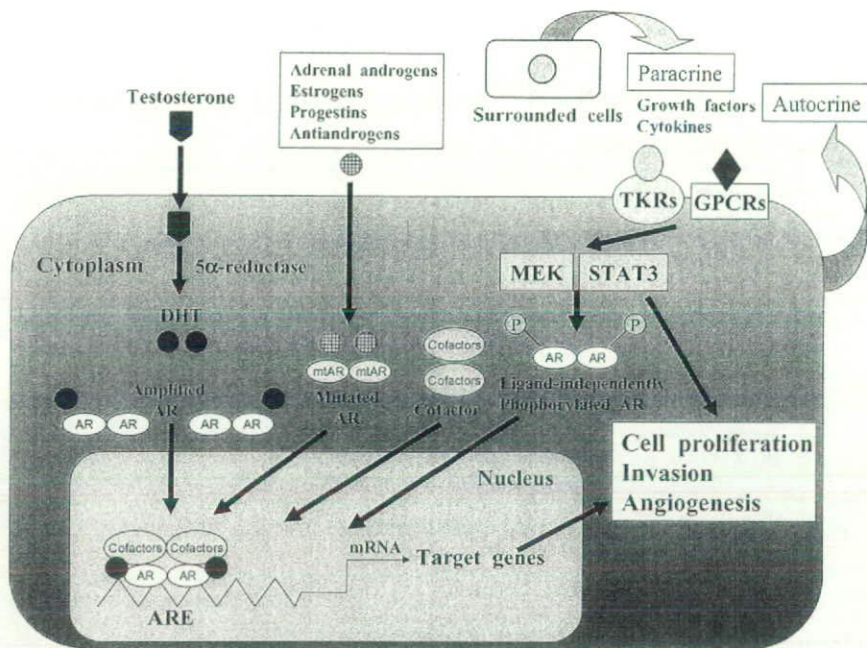


Fig. 1 Putative mechanisms of development of hormone-refractory prostate cancer (HRPC) cell. Androgen receptor (AR) activation such as amplification, mutation and the related AR signaling (AR binding to cofactors or ligand-independently phosphorylated AR) plays a role in the change from being hormone dependent to hormone independent. Additionally, growth factors and cytokines secreted in an autocrine or paracrine loop are considered to be involved in the development of HRPC.

characterized in prostate cancer progression, especially in the development of HRPC (Fig. 1). For example, epidermal growth factor (EGF) and its related family members are expressed in prostate cancer cells. It is reported that EGF and its receptor (EGF-R) are expressed in prostate cancer tissue, and are associated with disease-free survival.¹⁵⁻¹⁷ Furthermore, other growth factors, such as vascular endothelial growth factor (VEGF)^{18,19} and insulin-like growth factor (IGF),²⁰ are involved in prostate cancer progression.

Various cytokines are also considered to be important for prostate cancer progression. In particular, interleukin-6 (IL-6) can promote AR activity without androgen,²¹ through a mechanism involving signal transducer and activator of transcription 3 (STAT3) or mitogen activated protein kinase (MAPK) activation.^{22,23} Another mechanism is that IL-6 induces AR expression itself and leads to enhancement of androgen-responsive gene expression.²⁴ The fact that interleukin-8 (IL-8) plays an important role in the proliferation of prostate cancer cells has been confirmed by *in vitro* and *in vivo* studies; IL-8 stimulation accelerates prostate cancer tumorigenesis, angiogenesis and metastasis, and IL-8 especially has the potential for promotion of HRPC progression.^{25,26} In addition to IL-6 and IL-8, other cytokines (e.g. IL-4 and IL-10) are also related to HRPC.²⁷ Taking these findings together, many growth factor signals, cytokine signals and AR signals are not only independently regulated, but also interplay with each other. Hence, inhibition of these growth factors and/or their signals is expected to provide new therapy for prostate cancer, especially HRPC.

Classical RAS and local RAS

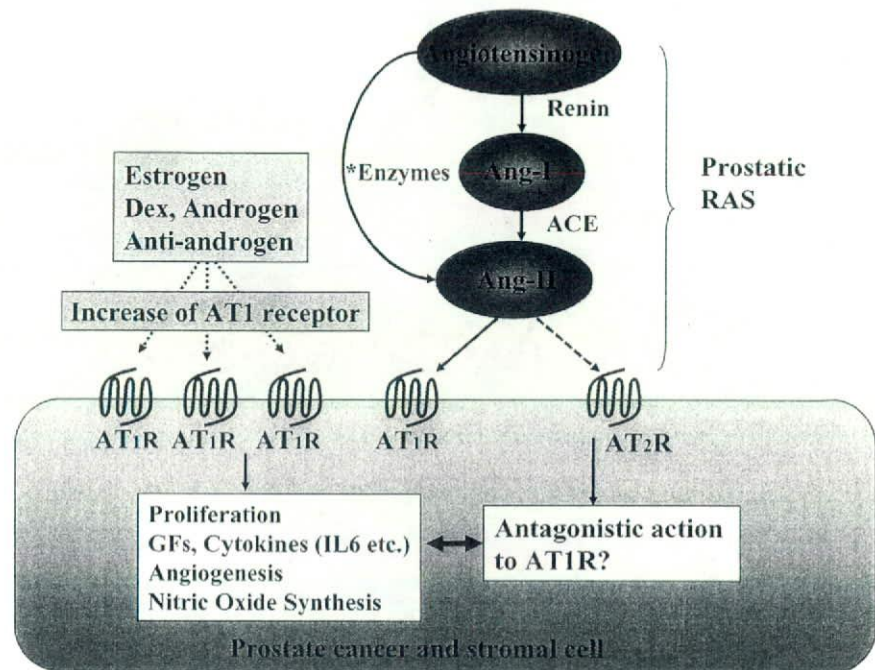
The renin-angiotensin system (RAS) has classically been identified in reno-cardiovascular organs including the kidney, heart and vessel walls, where its enzymatic actions and produced peptides are released systemically, leading to blood pressure regulation and electrolyte and fluid homeostasis. Ang-II, the main bioactive product of RAS, is spliced from the precursor substrate (angiotensinogen) by renin, and the decapeptide angiotensin (Ang-I) is cleaved by ACE. Ang-II exerts

biological activity by binding to two types of receptors; type 1 (AT1) and type 2 (AT2).^{28,29} Both receptors are functionally distinct polypeptides, with 30% sequence homology, and with seven transmembrane domains, belonging to the guanosine phosphate binding protein coupled receptor (GPCR) family.

Current investigations of RAS have shifted from its endocrine role to its autocrine and paracrine role in specific tissues, associated with tissue growth and/or differentiation (Fig. 2). Also, recent studies have revealed that Ang-II is a multifactorial effector, whose effects include vasoconstriction, release of aldosterone, facilitation of sympathetic transmission, and trophic actions on vascular smooth muscle, cardiac myocytes and fibroblasts.³⁰⁻³⁴ As well as the local RAS of the cardiovascular system, early studies have provided a body of evidence for a local RAS in other organs by identification of key RAS components: angiotensinogen, the AT1 receptor and renin. For instance, a functioning RAS has been identified in cardiovascular organs,³⁵ adipose tissue,³⁶ brain,³⁷ kidney,³⁸ pancreas,³⁹ ovary,⁴⁰ prostate⁴¹ and placenta.⁴² Recently, regulation of RAS has been shown to be complicated; for instance, renin is not always required for the generation of Ang-II from angiotensinogen because alternative enzymes can generate Ang-II (Fig. 2).⁴³⁻⁴⁵

The AT1 receptor binds to Ang-II with concentration-dependent affinity. In prostate cancer, there have been few reports besides our previous report in which AT1 receptor expression was shown to be higher in cancer tissue than in normal prostate tissue by reverse transcription-polymerase chain reaction (RT-PCR) analysis.⁸ Further comparative investigations of AT1 receptor expression have confirmed higher expression in breast cancer,⁴⁶ laryngeal carcinoma,⁴⁷ pancreatic cancer,⁴⁸ and choriocarcinoma⁴⁹ than in normal tissue. On the other hand, the constructional characteristics show that the AT2 receptor is composed of 363 amino acids with similarity to the AT1 receptor.⁵⁰ Interestingly, the antagonistic biological function of the AT1 receptor is mediated through the AT2 receptor. For instance, the AT2 receptor exerts vascular effects of vasodilatation, proliferation, differentiation and apoptotic effects in endothelial cells, mesangial cells and pheochromocytoma cell lines.⁵¹

Fig. 2 Local renin-angiotensin system in prostate. Angiotensin II (Ang-II) generated in the prostate gland influences the cancer physiology and pathology through the Ang-II type-1 (AT1) or type-2 (AT2) receptor. Besides the classical converting enzymes of this system, for example, renin and angiotensin converting enzyme (ACE), other enzymes (*enzymes: chymase, chymostatin-sensitive Ang II-generating enzyme (CAGE), cathepsin G, tissue type plasminogen activator (tPA), elastase, and tonin) convert angiotensinogen directly to Ang-II. Steroid hormones and anti-androgens augment AT1 receptor expression. Binding of Ang-II to the AT1 receptor exerts proliferative effects on prostate cancer and stromal cells, and binding to the AT2 receptor presumably exerts an antagonistic action to that of the AT1 receptor.



ACE inhibitors and ARBs

ACE inhibitors, which inhibit stimulation by Ang-II by decreasing its production, were developed as first-line drugs for hypertension and are widely used clinically. Recently, much evidence has accumulated that ACE inhibitors have inhibitory potential against cancers, *in vitro* and *in vivo*. ACE inhibitors retard the growth of a wide variety of cultured cancer cells *in vitro*,^{52,53} and further have the potential to inhibit tumorigenesis and angiogenesis induced in cancers in animal models *in vivo*.⁵³⁻⁵⁵ As mentioned above, some groups have reported clinical evidence that long-term Ang-II blockade by an ACE inhibitor may have a protective effect against cancer, and suggested that it could prevent carcinogenesis. These reports support the hypothesis that Ang-II accelerates carcinogenesis, and blockade of Ang-II stimulation has inhibitory potential against carcinogenesis.

Numerous orally active, selective AT1 receptor antagonists (ARBs) have been synthesized and available for the treatment of hypertension since the 1990s.^{56,57} Losartan, valsartan, irbesartan, eprosartan, telmisartan, and candesartan were approved by the Food and Drug Administration in 2000, and novel selective ARBs have now been developed. They have high affinity for AT1 receptors and almost no affinity for AT2 receptors. Their binding to AT1 receptors is competitive, with very slow dissociation. They dose-dependently block the response to exogenous Ang-II.⁵⁸ ARBs share the same mechanism of action; however, they have different pharmacokinetic profiles. Besides lowering elevated blood pressure, ARBs have further beneficial actions including amelioration of vascular diseases, postmyocardial infarction remodeling, and preservation of renal function in nephropathy.⁵⁹⁻⁶¹ Furthermore, ARBs are likely to have beneficial clinical effects on cancer cells, as well as ACE inhibitors.

ARBs and prostate cancer

To date, we have reported the potential of ARBs as novel therapeutic agents for HRPc.⁸ First, we evaluated the expression of AT1 receptor in

prostate cancers, which showed that the AT1 receptor mRNA was expressed in both prostate cancer and adjacent normal prostate tissue obtained from patients who underwent radical prostatectomy. RT-PCR analysis indicated that the AT1 receptor mRNA level tended to be increased in tumors compared with normal tissue. AT1 receptor mRNA was also recognized in human prostate cancer cell lines, LNCaP and DU145. In LNCaP, Ang-II stimulation induced tyrosine-phosphorylation of proteins through stimulation by EGF, which has potency to accelerate tumorigenesis of prostate cancer. Signal transducers such as MAPK and STAT3 were also activated immediately after stimulation with Ang-II. Additionally, candesartan suppressed not only activation of MAPK and STAT3 induced by Ang-II stimulation, but also their activation induced by EGF or IL-6 stimulation, as shown in Figure 3. Finally, candesartan suppressed the growth of LNCaP and DU145 cells induced by EGF. As in vascular endothelial cells,⁶² the AT1 receptor can transactivate the EGF-R, leading to activation of MAPK, STAT3 and protein kinase C (PKC) in cancer cells.⁶³ Although activation of MAPK is not always caused by only cell growth stimulants, the inhibition of cell growth using anticancer drugs most commonly requires inactivation of the MAPK and STAT3 pathways. Considering that ARBs can inhibit activation of MAPK and STAT3 through the AT1 receptor, an ARB could be a molecular targeting agent for use as an anticancer drug.

In *in vivo* experiments, we investigated the effect of candesartan on tumor xenografts of DU145 cells in athymic nude mice. We confirmed that there was a statistically significant difference in tumor relative volume between control (non-treated) and candesartan-treated mice at 4 weeks. Immunohistochemical staining showed a highly statistically significant difference in microvessel number in xenografts between control and candesartan-treated mice.⁸

Angiotensin and prostate stromal cells

From the viewpoint of growth factors and cytokines in prostate cancer, it is important to understand the autocrine and paracrine mechanisms

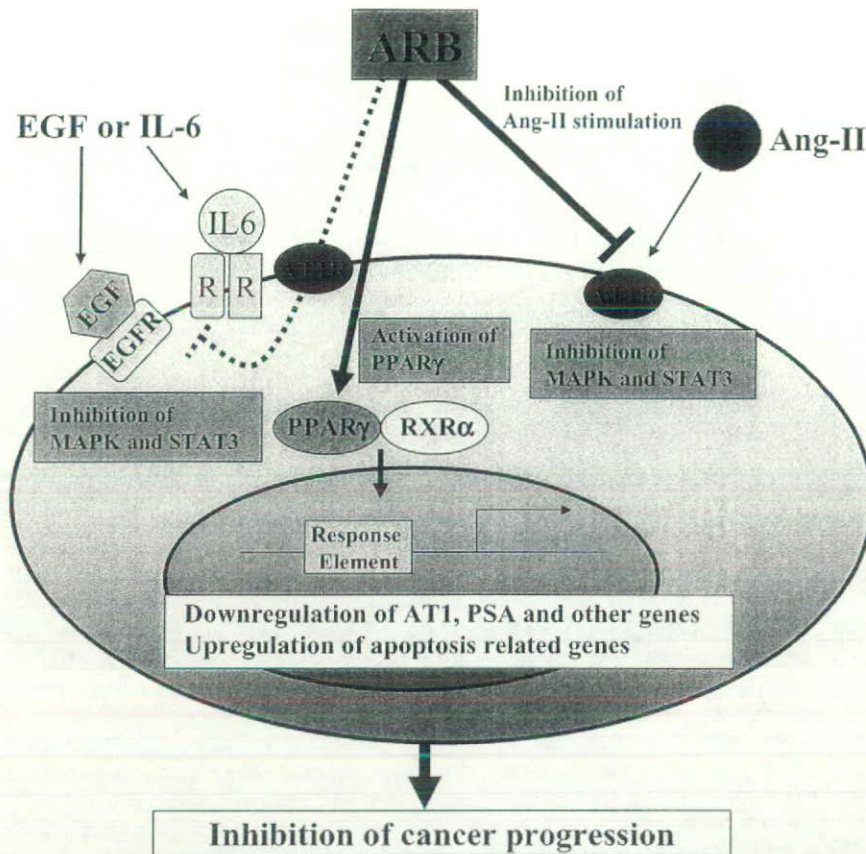


Fig. 3 Mechanism of AT1 receptor blocker (ARB) at multiple sites in prostate cancer cells. The ARB inhibits angiotensin II (Ang-II) binding to the AT1 receptor, and suppresses mitogenic actions by interaction with signal transduction via interleukin-6 (IL-6) or epidermal growth factor (EGF) receptors in prostate cancer cells. Telmisartan, an ARB, can bind to peroxisome proliferators activated receptor (PPAR γ), influencing various transcriptional activities in the regulation of multiple genes.

surrounding cancer cells. Many reports revealed an elevated serum IL-6 level in patients with HIRPC, and this cytokine is therefore thought to be involved in the progression of prostate cancer. Lee *et al.* reported that overexpression of IL-6 rendered androgen-sensitive prostate cancer cells more resistant to apoptosis induced by androgen deprivation.⁶⁴ As other growth factors and cytokines involved in the progression of prostate cancer, EGF, tumor necrosis factor α (TNF α), heparin-binding EGF (HB-EGF) and IGF⁶⁵ were shown to be expressed in stromal tissue.

Several recent studies have indicated that prostate stromal cells contain the AT1 receptor,⁶⁶ and as we previously demonstrated, the prostate stromal cell number was increased by Ang-II treatment.⁶⁷ It is well known that prostatic stromal cells, especially fibroblasts, are involved in the development of HIRPC accompanied by the secretion of several growth factors.^{17,68-70} We have confirmed that Ang-II induced the secretion of IL-6 and other cytokines including IL-1 α , IL-8 and MCP-1 from prostatic stromal cells.⁶⁷ IL-1 α is required for *in vivo* angiogenesis and invasiveness of different tumor cells, and contributed to the production of VEGF and TNF α in tumor cells cocultured with peritoneal macrophages.⁶⁸ IL-8 confers androgen-independent growth and migration of LNCaP cells through activation of the AR, without androgen stimulation.⁷¹ Ohta *et al.* reported that MCP-1 mRNA was expressed in gastric carcinoma, and its expression was significantly correlated with VEGF level.⁷² These factors secreted from PrSC stimulated by Ang-II treatment may contribute to the mechanisms underlying androgen independence through multiple pathways. It is therefore conceivable that Ang-II might induce neovascularization through activation of angiogenic factors via reactive prostate stroma, and specific

ARBs possibly inhibit carcinogenesis through suppression of angiogenesis.

Clinical efficacy of ARBs for prostate cancer

ARBs possessing these experimental demonstrations may offer clinical efficacy in patients with HIRPC. We conducted a pilot clinical study to examine whether an ARB was able to elicit an antiproliferative effect on HIRPC clinically.⁷³ Surprisingly, a quarter of patients with a prostate specific antigen (PSA) decline of more than 50% showed an improvement in performance status. We experienced some cases in which the PSA response was delayed for several months after starting ARB treatment. Thus, we presume that the administered dose of candesartan was too low to overcome multiple metastases. Furthermore, a possible cause of the delayed PSA decline after treatment is thought to be that an ARB functions as a molecular targeting or cytostatic agent at many targeted points of signal transduction through membrane receptors. ARBs have the ability to interact negatively with the phosphorylation of MAPK or STAT3 activated by EGF or IL-6 stimulation.⁸ They also have the potential to suppress the paracrine loop of growth factor or cytokine secretion from surrounding stromal tissue, which has been elucidated in vascular tissue and cardiac hypertrophy.⁷⁴ We also have confirmed a similar mechanism by which ARBs can suppress the cell growth and cytokine secretion of prostate stromal cells.⁶⁷ Based on these combined data, in advanced HIRPC cases with widespread metastases, a low dose of ARB, as usually given for hypertension, cannot stop disease progression completely, but might delay it. PSA

concentration rose coincident with the initiation of treatment, and several months later it declined or reached a stable state. Similar PSA kinetics were observed in a clinical trial using an angiogenesis inhibitor, TNP-470, for advanced HRPc.⁷⁵ Because both ARBs and TNP-470 are cytostatic rather than cytotoxic agents, they probably have slow PSA kinetics.

Some evidence suggesting that ARBs have antitumor potential against other solid tumors as well as prostate cancer has recently accumulated.⁷⁶⁻⁸⁰ If Ang-II and AT1 receptor have positive potency against tumorigenesis, two possible reasons that ARBs would have a stronger anticancer effect than ACE inhibitors are expected. The first is that angiotensin I is activated not only by an ACE-dependent pathway, but also by other enzymes such as chymase, chymostatin-sensitive angiotensin II-generating enzyme (CAGE), cathepsin G, tissue type plasminogen activator (tPA), elastase, and tonin, as shown in Figure 2. ACE inhibitors do not inhibit all Ang-II production, whereas ARBs theoretically can inhibit all AT1 receptor activation stimulated by Ang-II. The second is that blockade of the AT1 receptor induced by ARBs increases the bioavailability of Ang-II by reducing the inhibitory effect on renin secretion, producing upregulation and overstimulation of the AT2 receptor, which in turn potentiates antiproliferative effects.⁸¹⁻⁸⁴

At the end stage of HRPc, most patients develop a cachectic condition, in which they are constantly exposed to inflammatory cytokines produced by recurrent cancer cells. In other words, patients with HRPc suffer from severe chronic inflammation. Based on our clinical study using an ARB for patients with HRPc, ARBs may therefore be beneficial against inflammation caused by cancer. More interestingly, in our previous study, real-time RT-PCR analyses revealed that RAS components were expressed more highly in HRPc tissue than in normal and untreated prostate cancer tissue.⁴¹ Expression of the AT1 receptor was augmented in LNCaP cells treated with estrogen, dexamethasone and antiandrogen (Fig. 2), which are generally used as hormonal therapy for patients with prostate cancer.⁴¹ It is thus speculated that recurrent prostate cancer has greater susceptibility to ARBs compared to normal or untreated prostate cancer. Indeed, we confirmed the beneficial effect of an ARB to inhibit PSA progression in advanced HRPc patients.⁷³ Thus, these observations satisfactorily support the hypothesis of high expression of RAS components, especially the AT1 receptor, in recurrent prostate cancer tissue.

ARBs and their structures

Since DuPont pharmaceutical company discovered DuP753 (losartan), many pharmaceutical companies consequently developed usable ARBs with greater selectivity for the AT1 receptor than losartan, which have been used worldwide for hypertension. Earlier studies and our group have revealed the biological effects of ARBs on prostate cancer cells using candesartan or other ARBs such as losartan. Most of these ARBs are structurally similar to losartan and are biphenyl tetrazole derivatives, except one ARB, telmisartan. Telmisartan is structurally quite distinct from all other ARBs: it is a highly lipid-soluble, non-tetrazole derivative, with a single carboxylic acid group instead of the large tetrazole ring, as a result showing an extraordinarily high volume of distribution compared to other ARBs.⁸⁵

A recent investigation has demonstrated that telmisartan bears a structural resemblance to pioglitazone, a thiazolidinedione ligand of peroxisome proliferators activated receptor γ (PPAR γ),⁸⁶ which has been used widely for the treatment of type 2 diabetes. PPAR γ is substantially recognized as a member of the nuclear hormone receptor superfamily that exerts transcriptional activity on the regulation of multiple genes involved in lipid metabolism, inflammation and steroid

hormones.⁸⁷⁻⁹² Interestingly, activation of this receptor by various ligands induces apoptosis in several kinds of cancer. In particular, prostate cancer cell lines including LNCaP, PC-3 and DU145 cells express this receptor, and PPAR γ ligands influence cell growth accompanied by a decrease in PSA secretion.⁹³ Human prostate cancer tissue also expresses this receptor.^{94,95}

Effects of ARBs on prostate cancer through PPAR γ

An earlier report indicated that telmisartan can function as a partial agonist of PPAR γ , and influences the expression of PPAR γ -targeted genes.⁸⁶ When the ability of ARBs to activate PPAR γ was measured in a transient transfection assay, telmisartan similarly showed the highest activity toward PPAR γ by about 15-fold compared to losartan.⁸⁵ It should be noted that telmisartan belongs to a species of selective PPAR γ modulators (SPPARMs).^{96,97} Conventional PPAR γ activators such as pioglitazone typically function as full agonists of the receptor and affect the expression of various genes, while SPPARMs function as partial agonists, with limited effects on gene expression.^{86,97} Indeed, we confirmed that telmisartan did not have full binding ability as do PPAR γ agonists, and it has the potential to bind to PPAR γ of at least one third the ability of troglitazone.⁹³

These observations support the hypothesis that telmisartan, as a SPPARM, not only blocks the Ang-II receptor, but also weakly activates the PPAR γ receptor, leading to regulation of carbohydrate and lipid metabolism. Although most ARBs that have no potential as a PPAR γ ligand affect the cell proliferation of prostate cancer, only telmisartan is considered to affect the cell growth of prostate cancer containing PPAR γ through both ARB's biological function and PPAR γ activation synergistically (Fig. 3).

More interestingly, an *in vivo* study demonstrated that telmisartan significantly improved insulin sensitivity without weight gain in fat-fed mice.⁹⁷ Also, clinical studies showed that PPAR γ -modulating ARBs were capable of retaining the beneficial metabolic PPAR γ effects, which was associated with circumvention of adverse effects such as weight gain and systemic edema.⁹⁸ These observations suggest that SPPARMs like telmisartan may provide a new therapeutic option for prostate cancer patients suffering from hormonal therapy-induced obesity or hyperglycemia.

Functions of ARBs as GPCR antagonist in prostate cancer

Angiotensin II receptors, the AT1 and AT2 receptors, are members of the GPCR family. All GPCRs have a common central core domain consisting of seven transmembrane helices connected by three intracellular and three extracellular loops. In general, GPCRs are activated by a variety of ligands, including hormones, peptides, growth factors, and neurotransmitters, and play critical roles in many physiological and pathological processes, for example, development, angiogenesis, and inflammation. Current investigations have demonstrated that many GPCRs and their ligands are also involved in cancer progression.

The expression of some GPCRs, including those receptors for lysophosphatidic acids,⁹⁹ endothelin-1,^{100,101} and bradykinin,¹⁰² has been reported to be observed in normal prostatic epithelium and in prostate cancer cells. Interestingly, endothelin-1 is one of the peptides related to vasoconstriction, and furthermore, the expression level of its receptor was more significantly increased in advanced androgen-independent prostate cancer than in localized cancer.¹⁰⁰ It should be noted that the phenomena of endothelin-1 are similar to those of angiotensin II, as we

previously demonstrated. Additionally, Xu *et al.* indicated that elevated levels of the orphan GPCR, prostate specific G protein coupled receptor, were associated with the progression of prostate cancer.¹⁰⁵ Hence, some GPCRs and their ligands have been considered to be involved in the development and progression of prostate cancer.

The unique mechanical feature of GPCRs is so-called transactivation, which activates a receptor tyrosine kinase independent of exogenous addition of its specific ligand. ARBs and other blockers of GPCRs presumably have the potential for 'negative' transactivation. For example, as described above, candesartan inhibited cell proliferation through the AT1 receptor and its interplay in cell signaling induced by EGF or IL-6 (Fig. 3).⁸ Given that specific GPCRs expressed in prostate cancer cells will be identified, this could lead to the development of novel therapeutic products for prostate cancer, especially hormone-refractory cancer. Although side-effects could be a concern because GPCRs are generally ubiquitous, ARBs are likely to have few side-effects; therefore, ARBs could be easily used for patients with advanced prostate cancer.

Conclusion and future directions

ARBs have the potential to inhibit the growth of prostate cancer and stromal cells through the AT1 receptor. In particular, these drugs elicit multifactorial changes in cell proliferation, angiogenesis and fibrogenesis in cancer tissue. The action of ARBs against tumor cells is cytostatic, but not cytotoxic, indicating that these drugs are so-called molecular targeting medicine. Investigation of how drugs such as ARBs have efficacy in HRPc will contribute to elucidation of the molecular mechanisms of the progression from hormone-dependent to hormone-independent prostate cancer. Furthermore, ARBs have actions not only in cancer cells (epithelial cells), but also in stromal cells, suggesting the possibility that they are also effective for the chemoprevention of prostate cancer or the development of benign prostatic hypertrophy.

Acknowledgments

The original studies in our program were supported by an Umehara Grant from the Yokohama Medical Foundation, a grant for the 2007 Strategic Research Project (No. K19028) of Yokohama City University, a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Science and Technology, and a COE Research Grant from the Japan Society for the Promotion of Science.

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4-Vinyl-2,6-dimethoxyphenol (canolol) suppresses oxidative stress and gastric carcinogenesis in *Helicobacter pylori*-infected carcinogen-treated Mongolian gerbils

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Oxidative stress is linked to gastric carcinogenesis because of its ability to damage DNA. Here we examined antioxidative and anti-inflammatory effects of 4-vinyl-2,6-dimethoxyphenol (canolol), a recently identified potent antioxidative compound obtained from crude canola oil, on *Helicobacter (H.) pylori*-induced gastritis and gastric carcinogenesis using a Mongolian gerbil model. The animals were allocated to *H. pylori*-infection alone (12 weeks) or *H. pylori* + *N*-methyl-*N*-nitrosourea (MNU) administration (52 weeks). After oral inoculation of *H. pylori*, they were fed for 10 and 44 weeks with or without 0.1% canolol. *H. pylori*-induced gastritis, 5'-bromo-2'-deoxyuridine (BrdU) labeling and scores for cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) immunohistochemistry were attenuated in the canolol-treated groups. Expression of interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), COX-2 and iNOS mRNA in the gastric mucosa, and serum 8-hydroxy-2'-deoxyguanosine (8-OHdG), anti-*H. pylori* IgG and gastrin levels were also significantly lower in canolol-treated groups. Furthermore, the incidence of gastric adenocarcinomas was markedly reduced in the *H. pylori* + MNU + canolol-treated group [15.0% (6/40)] compared to the control group [39.4% (13/33)] ($p < 0.05$). These data indicate canolol to be effective for suppressing inflammation, gastric epithelial cell proliferation and gastric carcinogenesis in *H. pylori*-infected Mongolian gerbils. Interestingly, the viable *H. pylori* count was not changed by the canolol containing diet. Thus, the data point to the level of inflammation because of *H. pylori* rather than the existence of the bacteria as the determining factor. Importantly, canolol appears to suppress induction of mRNAs for inflammatory cytokines.

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Key words: canolol; antioxidant; canola oil; *Helicobacter pylori*; Mongolian gerbils

Helicobacter pylori (*H. pylori*) is now considered as the most important etiological agent for chronic gastritis and peptic ulcer disease, as well as a cause of gastric carcinoma.^{1,2} There is accumulating evidence that eradication of *H. pylori* in the stomach by administration of oral antimicrobial agents results in the resolution of *H. pylori*-infected chronic active gastritis and peptic ulceration and significantly lowers the risk of stomach tumor development in patients without precancerous lesions.^{3,4} However, such bacterial eradication treatment has not been always successful. The occurrence of antibiotic-resistant *H. pylori* has been reported, and it is occasionally associated with adverse effects.⁵ Therefore, it is still desirable to develop alternative approaches for cancer prevention and a number of studies have demonstrated protective effects of plant extracts against *H. pylori* infection, such as green tea catechins⁶ and a garlic extract.⁷ In a previous study, we also found fruit-juice concentrate of Japanese apricot (ume) (CJA) to decrease the number of *H. pylori* and suppress chronic active gastritis in gerbils,⁸ although the mechanism was unclear. *H. pylori* infection has been reported to cause production of H₂O₂ in AGS human gastric epithelial cells, which might contribute to carcinogenesis.⁹ Correa *et al.* reported antioxidants like vitamin C or vitamin E have protective effects against *H. pylori*-induced lesions due to their free radical scavenging activity.^{10,11}

4-Vinyl-2,6-dimethoxyphenol (canolol), was recently identified as a potent antioxidative compound in crude canola oil, exhibiting

more potent antialkylperoxyl [ROO*] radical activity than well-known antioxidants, like α -tocopherol, vitamin C, β -carotene, rutin and quercetin.¹² We previously reported strong scavenging capacity against the endogenous mutagen, peroxynitrite (ONOO⁻), and suppression of bacterial mutation, consistent with the earlier observed protection from DNA damage, and prevention of oxidation of lipids and proteins.¹³

The Mongolian gerbil (*Meriones unguiculatus*) provides a useful animal model of *H. pylori*-induced chronic active gastritis that allows investigation of morbidity-related pathological epithelial alterations in gastric mucosa, and their development into intestinal metaplasia and gastric neoplasia.¹⁴ The purpose of our study was to evaluate the effectiveness of canolol for inhibition of *H. pylori*-infected chronic gastritis and gastric carcinogenesis. An investigation of effects on induction of cytokines in mouse peritoneal macrophages *in vitro* was included.

Material and methods

Chemicals

Canolol, 4-vinyl-2,6-dimethoxyphenol (molecular weight, 180) (Fig. 1), is a novel and potent antioxidant, contained in crude canola oil. In our study, it was synthesized to at least 95% purity (confirmed by nuclear magnetic resonance) and mixed with 2,6-di-*tert*-butyl-4-methylphenol (butylhydroxytoluene, BHT, Sigma Chemical, St Louis, MO) at final concentration of 300 ppm at Junsei Chemical, Tokyo, Japan. The preparation was sealed under helium or nitrogen, and also kept as an ethanol stock solution in nitrogen at -80°C.

Diets

AIN93G was purchased from Clea Japan, (Tokyo, Japan). Tert-butylhydroquinone (tBHQ), contained in the standard recipe of AIN93G, was excluded to facilitate analysis of subtle changes because of canolol. Soybean oil was replaced with canola oil containing 443.7 ppm tocopherol (158.7 ppm α -tocopherol, 279.6 ppm γ -tocopherol, 5.4 ppm δ -tocopherol and no β -tocopherol) (Showa Sangyo, Funabashi, Japan). Canolol powder containing 300 ppm BHT was dissolved in ethanol and mixed with the modi-

This article contains supplementary material available via the Internet at <http://www.interscience.wiley.com/jpages/0020-7136/suppmat>.

Grant sponsor: Ministry of Health, Labour and Welfare, Japan (Third-term Comprehensive 10-year Strategy for Cancer Control); Grant number: H19-ippan-013. Grant sponsor: Ministry of Education, Culture, Science, Sports and Technology of Japan; Grant number: 18590395.

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Received 25 July 2007; Accepted after revision 25 September 2007

DOI 10.1002/ijc.23245

Published online 4 December 2007 in Wiley InterScience (www.interscience.wiley.com).

fied AIN93G described earlier to give a final concentration of 0.1% canolol and 0.3 ppm BHT. Since no information was available regarding *in vivo* administration of canolol, previous literature for other antioxidants was referenced for a suitable dose; 0.1–0.5% tocopherol successfully suppressed formation of colon aberrant crypt foci¹⁵ and mammary adenocarcinomas.¹⁶ Since canolol was revealed to be a more potent oxidative radical scavenger than tocopherol,¹³ the lowest concentration (0.1%) was applied here. For controls, 0.3 ppm BHT alone or no antioxidant supplement in the basal diet were used. The diets sealed under vacuum were stored in a freezer at -30°C and thawed for use everyday. Leftovers were measured on the next day and the new diet supplied given again to prevent excessive oxidation.

Carcinogen

N-methyl-*N*-nitrosourea (MNU) (Sigma Chemical, St Louis, MO) was dissolved in distilled water at the concentration of 10 ppm and administered *via* light-shielded bottles in drinking water *ad libitum*. MNU solutions were freshly prepared 3 times per week.

Inoculation of *H. pylori*

ATCC43504 (American Type Culture Collection, Manassas, VA) was grown in Brucella broth (Becton Dickinson, Cockeysville, MD), containing 7% (v/v) heat-inactivated fetal bovine serum, at 37°C under microaerophilic conditions, at high humidity for 24 hr. After 24 hr fasting, gerbils were inoculated *via* an oral catheter with 1.0 ml aliquots of *H. pylori* culture containing 1.0×10^8 colony-forming units/ml of the organisms. Four hours later, the animals were again allowed free access to food.

Animals and experimental protocol

One hundred seventy-eight specific-pathogen-free male Mongolian gerbils (MGS/Sea; Seac Yoshitomi, Fukuoka, Japan), 6 weeks old, were used in this study. They were housed in an air-conditioned biohazard room designed for experimentally infected animals, with a 12-hr light/12-hr dark cycle and were allowed free access to food. The experimental design is illustrated in Figure 2. The animals were allocated to Experiments I or II.

In Experiment I, 58 gerbils were divided into 6 groups (A–F): [Group A, $n = 10$]: *H. pylori*-infected, canolol + BHT-treated animals; [Group B, $n = 10$]: *H. pylori*-infected, BHT-treated animals, no canolol; [Group C, $n = 10$]: *H. pylori*-infected, untreated

animals; [Group D, $n = 10$]: Broth-inoculated (no *H. pylori*), canolol + BHT-treated animals; [Group E, $n = 10$]: Broth-inoculated, only BHT-treated animals; and [Group F, $n = 8$]: Broth-inoculated, untreated animals. Animals were killed at 18 weeks of age for midterm examination.

In Experiment II, 120 gerbils were divided into 4 groups (G to J). Two weeks after inoculation of *H. pylori*, Groups G, H and I were administered with MNU for 20 weeks. Group J was given broth and autoclaved distilled water as control. Groups G, H, I and J were given, (i) canolol + BHT, (ii) BHT, (iii) control diet and (iv) canolol + BHT diet, respectively, from the 8th experimental week to the end of the experimental period. BrdU at a dose of 100 mg/kg was injected intraperitoneally 60 min before the sacrifice at experimental week 52. The study was approved by the Animal Care Committee of Aichi Cancer Center Research Institute.

Bacterial cultures

To assess bacterial colonization in the gastric mucosa, half of each glandular stomach from gerbils in Experiment I was homogenized with 1.0 ml of phosphate buffered saline (PBS) for culture of *H. pylori*. One hundred-microliter aliquots were inoculated onto *H. pylori* agar plates (Nissui Pharmaceutical, Tokyo, Japan), which were then incubated at 37°C under microaerophilic conditions. The numbers of *H. pylori* colonies were counted after 5–7 days.

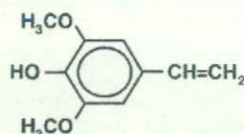


FIGURE 1 – Structure of 4-vinyl-2,6-dimethoxyphenol (canolol). Molecular weight, 180.

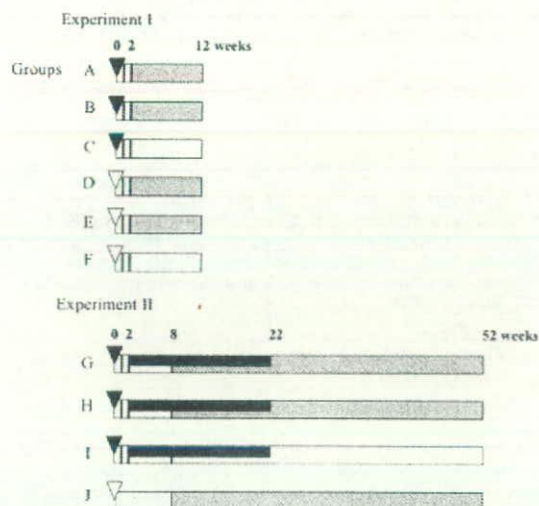


FIGURE 2 – Experimental design. Animals, 6-week-old male Mongolian gerbils; ▽, *H. pylori* inoculation (i.g.); ▽, Broth only, control. ■, MNU in drinking water at the concentration of 10 ppm. ▨, 0.1% canolol/0.5 ppm 2,6 Di-tert-butyl-4-methylphenol (BHT)/AIN93G diet; □, 0.5 ppm BHT/ AIN93G diet, control; □, AIN93G diet, control.

TABLE 1 – PCR PRIMERS USED FOR LIGHTCYCLER ANALYSIS

Gene	Sequence	Product size (bp)
GAPDH	F: 5'-AACGGCACAGTCAAGGCTGAGAACG-3'	118
	R: 5'-CAACATACTCGGCACCGGCATCG-3'	
IL-1 β	F: 5'-TGACTTCACCTTGAATCCGTCTCT-3'	91
	R: 5'-GGCAACAAGGGAGCTCCATCAC-3'	
TNF- α	F: 5'-GCTGCCCCACCTCGTGCTC-3'	89
	R: 5'-CTTGATGGCAGACAGGAGGCTGACC-3'	
COX-2	F: 5'-GCCGTCGAGTTGAAAGCCCTCTACA-3'	97
	R: 5'-CCCCGAAGATGGCGTCTGGAC-3'	
iNOS	F: 5'-GCATGACCTTGGTGTGGGTGCC-3'	110
	R: 5'-GCAGCCTGTGTGAACCTGGTGAAGC-3'	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

TABLE II - HISTOPATHOLOGICAL RESPONSES IN GERBILS

Experiments	Groups	No.	Treatments	Antrum						Corpus													
				Infiltration of neutrophils	Infiltration of mononuclear cells	Hyperplasia	Intestinal metaplasia	BrU labeling index (%)	Score of COX-2 immunohistochemistry	Score of iNOS immunohistochemistry	Infiltration of neutrophils	Infiltration of mononuclear cells	Hyperplasia	Intestinal metaplasia	BrU labeling index (%)	Score of COX-2 immunohistochemistry	Score of iNOS immunohistochemistry						
Experiment I	A	10	<i>Hp</i> -> Canolol + BHT	1.2 ± 0.4 ¹	2.2 ± 0.4 ¹	1.2 ± 0.6 ¹	0.0 ± 0.0	12.5 ± 2.5 ¹	0.9 ± 0.2 ¹	0.9 ± 0.1 ¹	Experiment II	H	33	<i>Hp</i> + MNU -> BHT	2.6 ± 0.3	3.0 ± 0.0	2.5 ± 0.3	1.7 ± 0.4	18.5 ± 5.4	1.7 ± 0.5	2.1 ± 0.3		
	B	10	<i>Hp</i> -> BHT	2.9 ± 0.3	3.0 ± 0.1	2.2 ± 0.3	0.0 ± 0.0	20.3 ± 3.7	2.2 ± 0.2	1.7 ± 0.2		I	36	<i>Hp</i> + MNU	2.7 ± 0.2	3.0 ± 0.0	2.6 ± 0.4	1.8 ± 0.4	20.6 ± 4.6	1.8 ± 0.5	1.7 ± 0.5		
	C	10	<i>Hp</i>	3.0 ± 0.1	3.0 ± 0.1	2.3 ± 0.4	0.0 ± 0.0	20.9 ± 3.3	2.3 ± 0.3	1.5 ± 0.3		J	5	Broth -> Canolol + BHT	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.6 ± 0.6	0.0 ± 0.0	0.2 ± 0.1		
	D	10	Broth -> Canolol + BHT	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	5.2 ± 0.8	0.2 ± 0.1	0.2 ± 0.2		Experiment II	I	36	<i>Hp</i> + MNU -> BHT	2.8 ± 0.3	3.0 ± 0.1	2.6 ± 0.3	1.6 ± 0.5	12.3 ± 2.9	1.2 ± 0.4	1.6 ± 0.5	
	E	10	Broth -> BHT	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	5.1 ± 0.8	0.3 ± 0.1	0.3 ± 0.1			J	5	<i>Hp</i> + MNU	2.7 ± 0.3	3.0 ± 0.2	2.8 ± 0.3	1.7 ± 0.3	13.8 ± 1.9	1.3 ± 0.4	1.4 ± 0.3	
	F	8	Broth	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 1.1	0.3 ± 0.1	0.2 ± 0.1			Experiment II	I	36	<i>Hp</i> + MNU -> BHT	2.2 ± 0.3	2.5 ± 0.3 ⁴	2.1 ± 0.5 ⁴	0.8 ± 0.3 ³	7.9 ± 2.4 ³	0.6 ± 0.3 ³	1.0 ± 0.4 ³
	G	40	<i>Hp</i> + MNU -> Canolol + BHT	1.9 ± 0.4 ³	2.3 ± 0.4 ³	1.9 ± 0.4 ³	0.9 ± 0.5 ³	10.4 ± 2.3 ³	1.0 ± 0.4 ³	1.2 ± 0.5 ³				J	5	Broth -> Canolol + BHT	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 0.7	0.0 ± 0.0	0.3 ± 0.2
Experiment I	A	10	<i>Hp</i> -> Canolol + BHT	0.9 ± 0.2 ¹	0.9 ± 0.2 ¹	0.7 ± 0.2 ¹	0.0 ± 0.0	6.8 ± 1.7 ²	0.6 ± 0.2 ²	0.5 ± 0.1 ²	Experiment II	H	33	<i>Hp</i> + MNU -> BHT	2.8 ± 0.3	3.0 ± 0.1	2.6 ± 0.3	1.6 ± 0.5	12.3 ± 2.9	1.2 ± 0.4	1.6 ± 0.5		
	B	10	<i>Hp</i> -> BHT	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.0 ± 0.0	9.6 ± 2.6	1.0 ± 0.2	0.8 ± 0.2		I	36	<i>Hp</i> + MNU	2.7 ± 0.3	3.0 ± 0.2	2.8 ± 0.3	1.7 ± 0.3	13.8 ± 1.9	1.3 ± 0.4	1.4 ± 0.3		
	C	10	<i>Hp</i>	1.5 ± 0.2	1.5 ± 0.2	1.2 ± 0.1	0.0 ± 0.0	10.4 ± 2.7	1.1 ± 0.1	0.8 ± 0.2		J	5	Broth -> Canolol + BHT	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 0.7	0.0 ± 0.0	0.3 ± 0.2		
	D	10	Broth -> Canolol + BHT	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	3.1 ± 0.6	0.2 ± 0.1	0.2 ± 0.1		Experiment II	I	36	<i>Hp</i> + MNU -> BHT	2.2 ± 0.3	2.5 ± 0.3 ⁴	2.1 ± 0.5 ⁴	0.8 ± 0.3 ³	7.9 ± 2.4 ³	0.6 ± 0.3 ³	1.0 ± 0.4 ³	
	E	10	Broth -> BHT	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	3.2 ± 0.4	0.2 ± 0.1	0.2 ± 0.0			J	5	<i>Hp</i> + MNU	2.7 ± 0.3	3.0 ± 0.2	2.8 ± 0.3	1.7 ± 0.3	13.8 ± 1.9	1.3 ± 0.4	1.4 ± 0.3	
	F	8	Broth	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 0.5	0.3 ± 0.1	0.3 ± 0.1			Experiment II	I	36	<i>Hp</i> + MNU -> BHT	2.2 ± 0.3	2.5 ± 0.3 ⁴	2.1 ± 0.5 ⁴	0.8 ± 0.3 ³	7.9 ± 2.4 ³	0.6 ± 0.3 ³	1.0 ± 0.4 ³
	G	40	<i>Hp</i> + MNU -> Canolol + BHT	2.2 ± 0.5 ³	2.5 ± 0.3 ⁴	2.1 ± 0.5 ⁴	0.8 ± 0.3 ³	7.9 ± 2.4 ³	0.6 ± 0.3 ³	1.0 ± 0.4 ³				J	5	Broth -> Canolol + BHT	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 0.7	0.0 ± 0.0	0.3 ± 0.2

Values for results are expressed as means ± SD.

¹*p* < 0.01 vs. Groups B and C; ²*p* < 0.05 vs. Groups B and C; ³*p* < 0.01 vs. Groups H and I; ⁴*p* < 0.05 vs. Groups H and I.

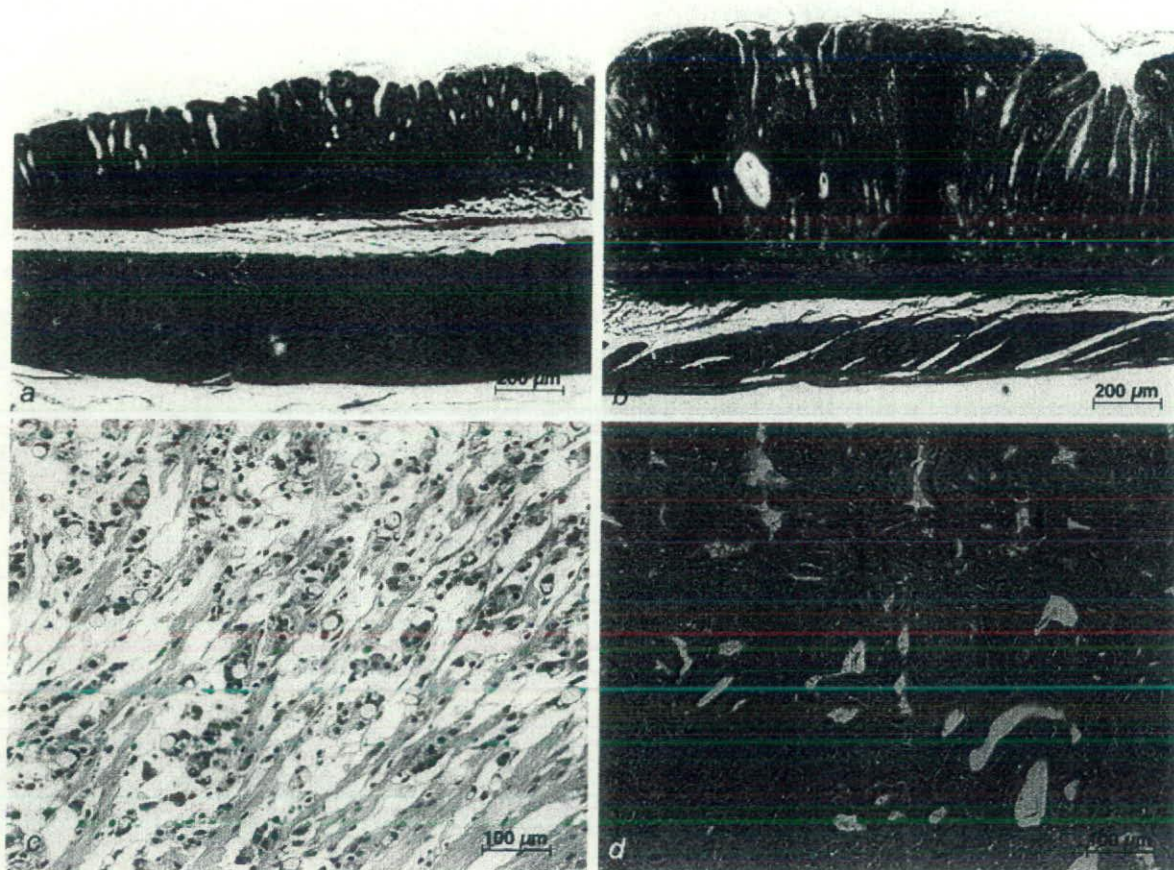


FIGURE 3 – Histopathological findings and histology of adenocarcinomas in the gastric mucosa. (a) Mild gastritis in a Group A gerbil at 12 weeks postinfection with canolol treatment. (H&E; $\times 25$); (b) Marked infiltration of inflammatory cells and hyperplasia is seen in a Group B gerbil with control diet (H&E; $\times 25$); (c) Signet-ring cell carcinoma at 52 weeks in a Group H gerbil (H&E; $\times 200$). (d) Well differentiated adenocarcinoma in a glandular stomach at 52 weeks in a Group I gerbil (H&E; $\times 80$).

Histopathology and immunohistochemistry

For histological and immunohistochemical examination, the stomachs were fixed in 10% neutral buffered formalin for 24 hr and embedded in paraffin. Serial paraffin sections (4- μm thick) were prepared and stained with hematoxylin and eosin (H&E) for morphological observation, and immunohistochemistry for iNOS and COX-2. Mucosal inflammation in the gastric mucosa was analyzed on H&E-stained sections. The inflammatory responses of glandular mucosa were graded according to the following morphological criteria: Grade 0 (normal), Grade 1 (mild), Grade 2 (moderate) and Grade 3 (marked) (Supplementary Table I). Tissue sections were immunostained for BrdU labeling with a mouse monoclonal anti-BrdU antibody (1:50, DAKO) as described previously.¹⁷ Labeling indices (LI) were calculated as the percentages of BrdU-positive epithelial cells within glands. For this purpose 10 different arbitrarily selected points in the antrum and corpus mucosa were selected for quantitation. Immunohistochemical analyses of COX-2 and iNOS were carried out as previously described,¹⁸ using an anti-COX-2 mouse monoclonal antibody (diluted 1:200; BD Biosciences, San Jose, CA) and an anti-iNOS mouse polyclonal antibody (diluted 1:500, EMD Biosciences, San Diego, CA). To quantitate the degree of staining, a grading system was employed with the following criteria: Grade 0, no immunor-

activity; Grades 1–3, increasing degrees of intermediate immunoreactivity and Grade 4, extensive immunoreactivity.¹⁹

Effects of canolol on production of nitric oxide (NO), interleukin-12 (IL-12) and tumor necrosis factor- α (TNF- α) in peritoneal macrophages from mice

Mouse peritoneal macrophages were obtained after intraperitoneal injection of 3 ml of 10% proteoseptone and Griess assays for nitrate/nitrite measurement were conducted to assess the production of NO. Amounts of IL-12 and TNF- α were measured with enzyme-linked immunosorbent assay (ELISA). Macrophages were incubated with 0–300 μM canolol and 10% FBS at 37°C, then 1.0 $\mu\text{g}/\text{ml}$ lipopolysaccharide (LPS) or 1.0 $\mu\text{g}/\text{ml}$ LPS plus 0.2 $\mu\text{g}/\text{ml}$ interferon- γ (IFN- γ) were added. After incubation for 20 hr, NO, IL-12 and TNF- α production from macrophages was measured.

Analysis of mRNA expression of cytokines by real time quantitative PCR

Total RNA was extracted from the antrum and corpus in the glandular stomach (Experiment I) or in the border region between the 2 regions (Experiment II), using a RNA extraction kit (Isogen,

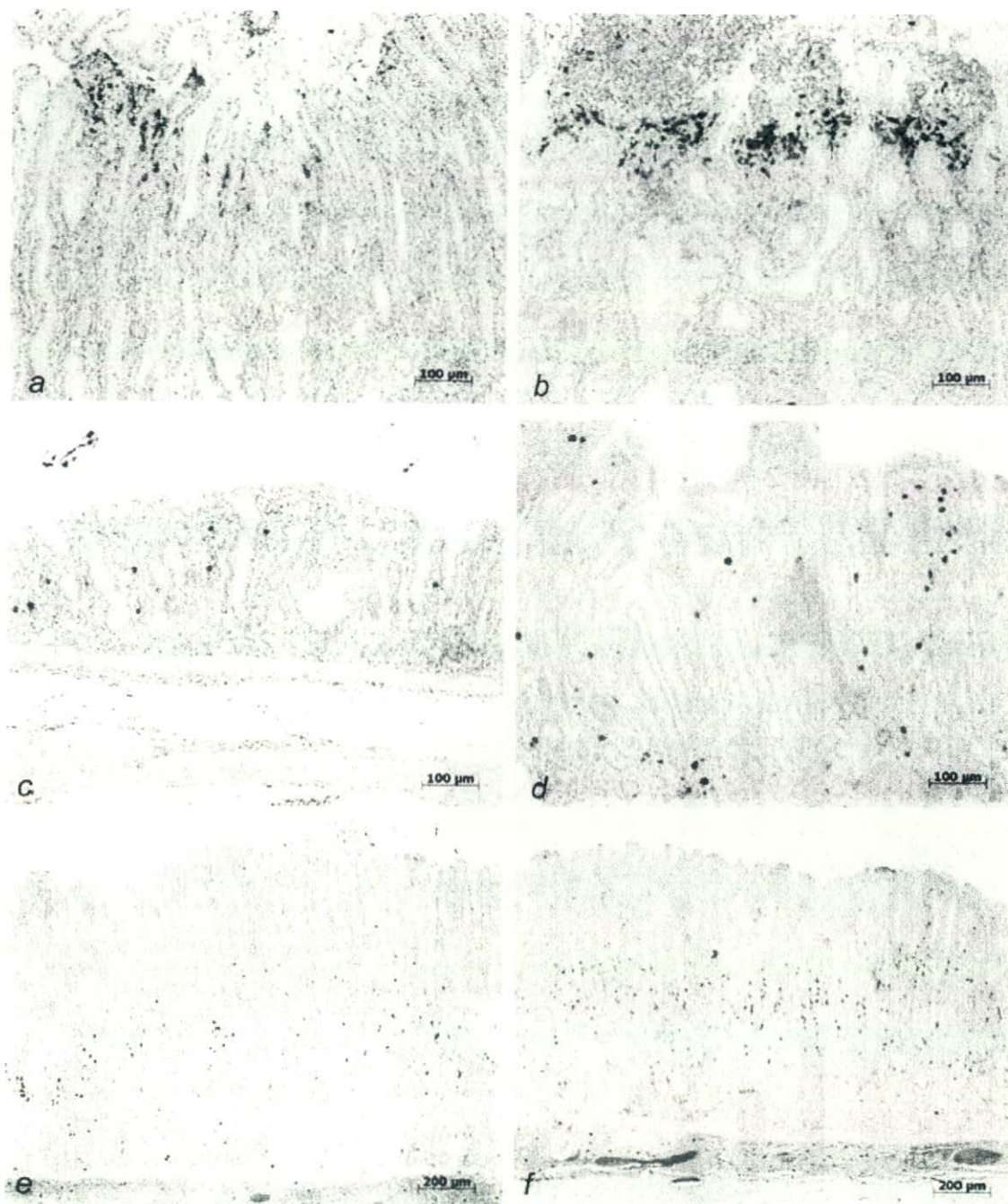


FIGURE 4 – Immunohistochemistry of gastric lesions. (a and b) COX-2. Original magnification, $\times 100$. (c and d) iNOS. Original magnification, $\times 100$. Note that the intensity of COX-2 and iNOS immunoreactivity in the canolol-treated gastric mucosa (a and c), is weaker than that in the control infection groups (b and d). (e and f) BrdU. BrdU positive cells are distributed more broadly in the control group (f) than the canolol-treated group (e). Original magnification, $\times 50$.

Nippon Gene, Tokyo, Japan). After DNase treatment, first strand cDNAs were synthesized using the ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. Quantitative PCR of IL-1 β , TNF- α , COX-2 and iNOS, was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), using gerbil-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal

control. The PCR was performed basically as described using a QuantiTect SYBR Green PCR (QIAGEN) kit with optimal Mg²⁺ concentration at 2.5 mM. The 5'- and 3'-primer sequences are listed in Table I. Specificity of the PCR reaction was confirmed using the melting program provided with the LightCycler software. To further confirm that there was no obvious primer dimer formation or amplification of any extra bands, the samples