Inhibition of Angiotensin II Activity Enhanced the Antitumor Effect of Cyclooxygenase-2 Inhibitors via Insulin-Like Growth Factor I Receptor Pathway

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ABSTRACT

Prostaglandin (PG) E2, a cyclooxygenase (COX) product, and angiotensin II are endogenous and have physiological roles in the body. On the other hand, an inducible isoform of COX (COX-2), insulin-like growth factor (IGF) II, and IGF-I receptor (IGF-IR) are up-regulated in colon carcinoma and might have crucial roles in tumor growth and invasion. The aim of the present study was to investigate the effects of COX-2 inhibitor and drugs blocking the biological activities of angiotensin II [angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs)] on IGF-IR expression and tumor growth in vivo. We also investigated the effects of PGE2, a major COX-2 product, in cancer cells and the effects of angiotensin II on IGF-IR expression and the underlying mechanism of action. In vitro studies, tumor growth and IGF-IR expression were investigated in Colon 26 cells inoculated in BALB/c mice. In vitro studies, the effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on IGF-IR expression were analyzed in three colon cancer cell lines (Colon 26, HCA-7, and LS174T). IGF-II-induced cell growth and invasion were analyzed inColon 26 cells in the presence and absence of NSAIDs (indomethacin and celecoxib) and angiotensin II. Celecoxib at the lowest effective dose for suppression of PG production (3 mg/kg) or an ACE inhibitor/ARB alone did not have a significant effect as compared with controls, although a high dose of celecoxib (>20 mg/kg) suppressed tumor growth. On the other hand, combination therapy with these two categories of drugs significantly reduced tumor growth in vivo. Treatment with both celecoxib and an ACE inhibitor/ARB decreased IGF-IR expression in inoculated tumor cells. In vitro studies, NSAIDs reduced IGF-IR expression in a dose-dependent manner in all three cell lines. NSAIDs also inhibited IGF-II-stimulated growth and invasion in a dose-dependent manner. PGE2 or angiotensin II treatment reversed the NSAID-induced down-regulation of IGF-IR expression, growth, and invasion. PGE2 and angiotensin II induced Akt phosphorylation, and LY294002 or wortmannin inhibited PGE2- or angiotensin II-induced IGF-IR expression, indicating that PGE2 and angiotensin II both regulate IGF-IR expression by the same Akt/phosphatidylinositol-3 pathway. Thus, combination therapy with NSAIDs and ACE inhibitors targeting IGF-II might be a novel and potentially promising strategy for the chemoprevention of colon cancer.

INTRODUCTION

Although advancements in the diagnosis and treatment of colorectal cancer have improved its prognosis, novel and more effective strategies for the prevention and cure need to be developed. NSAIDs have a chemopreventive effect (1–3). NSAIDs inhibit two COX isoforms, COX-1 and COX-2. Inhibition of COX-2 activity is thought to be one of the mechanisms by which NSAIDs exert their antineoplastic effects (4, 5). More than 85% of human colon cancers have increased levels of COX-2 when compared with adjacent normal tissue (6, 7). PGs derived from COX-2, such as PGE2, promote angiogenesis, growth, and invasiveness of colorectal cancer cells (8, 9). Therefore, COX-2 inhibitors are thought to be promising as a chemopreventive agent for colorectal cancer. Recent studies, however, indicate that even specific COX-2 inhibitors, as well as conventional NSAIDs, have potential side effects (10). Consequently, these agents should be used at the lowest dosage possible.

On the other hand, hypertension is a highly prevalent condition and is a common management problem in cancer patients (11). In a 9-year follow-up of a southern California community, systolic blood pressure was a significant predictor of subsequent cancer mortality (12). Angiotensin II is one of the most potent regulators of blood pressure (13), and it exerts a variety of physiological effects. It regulates circulating blood volume (13) and stimulates neovascularization (14) and cell proliferation (15). Renin, an enzyme that produces angiotensin, is found in cancer blood vessels (16), and long-term administration of ACE inhibitors, antihypertensive agents (17), reduces morbidity and mortality in cancer patients (18). These data indicate that angiotensin II might have an important role in carcinogenesis. Although ACE inhibitors are important candidates for cancer chemoprevention, the precise mechanism of action of angiotensin II on colon carcinogenesis is not known.

IGFs, especially IGF-II, are directly involved in tumorigenesis (19). Overexpression of IGF-II is observed in a large percentage of primary human colon cancers (20) and in several colon cancer cell lines (21). IGF-II exerts its mitogenic activity through the IGF-IR (19, 22). IGF-I or IGF-II might have an important role in carcinogenesis. Although ACE inhibitors are important candidates for cancer chemoprevention, the precise mechanism of action of angiotensin II on colon carcinogenesis is not known.

Among mechanisms regulating IGF-IR expression, the PI3/Akt pathway plays a crucial role in its expression (33). COX-2 activates the Akt pathway through PGE2 in colon cancer cells (34), indicating that COX-2/PGE2 is involved in IGF-IR expression. Angiotensin II activates the Akt pathway in vascular smooth muscle cells (35). Although it is not known whether angiotensin II is involved in PI3/Akt activation in colon cancer cells, it is suggested that angiotensin II induces IGF-IR expression through Akt activation. Therefore, we hypothesized that the simultaneous suppression of both COX-2 and angiotensin II might have valuable therapeutic effects against colon tumorigenesis in vivo.

The aim of the present study was to investigate the effects of
COX-2 inhibitors, especially at a low dosage, ACE inhibitors, and an ARB, alone or in combination, on tumor growth in a mouse allograft model. To clarify the underlying mechanism of action in \textit{in vivo} study, we also examined the effects of PGE$_2$, angiotensin II, and these inhibitors on IGF-IR expression and IGF-II-dependent cell proliferation and invasion. Furthermore, we examined whether Akt pathways are involved in PGE$_2$ and angiotensin II-related IGF-IR expression.

**MATERIALS AND METHODS**

**Cell Culture and Reagents.** Three colon cancer cell lines (Colon 26 cells, derived from a mouse colon cancer, and HCA-7 and LS174T cells, derived from a human colon cancer) were used. These cells were cultured in RPMI 1640 or DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FCS (JRH Biosciences, Lenexa, KS) and 1% antibiotics and antimycotics (Life Technologies, Inc., Grand Island, NY) in an atmosphere of 95% air and 5% CO$_2$ at 37°C. IGF-II was purchased from Pepro Tech (London, United Kingdom), and indomethacin and angiotensin II were obtained from Sigma Chemical Co. PGE$_2$ was purchased from Cayman Chemical (Ann Arbor, MI), and celecoxib was a kind gift from Pharmacia Corp. (Skokie, IL).

**Western Blotting and Antibodies.** Cells were harvested and lysed in radioimmunoprecipitation assay buffer [1% Triton X-100, 150 mM NaCl, 0.1 M Tris (pH 7.4), 0.02 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40] to obtain protein extracts. In the phosphorylated Akt expression experiment, the cells were harvested in boiling lysis buffer [1% SDS, 0.4 mM sodium orthovanadate, and 10 mM Tris (pH 7.4)]. Total protein was assayed using protein assay reagent (Pierce, Rockford, IL). Centrifuged lysates were denatured and separated on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose, and the filters were probed with the primary antibodies and developed using the eugenerogenated chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Antibodies against the β subunit of IGF-IR (sc-713), COX-1 (sc-1752), and COX-2 (sc-1746) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphorylated Akt (Ser-473) antibody (#9271) and Akt antibody (#9272) were obtained from Cell Signaling Technology (Beverly, MA). The experiments were performed in triplicate, and the results were presented only when they were reproducible.

**Tumor Growth in BALB/c Mice.** All animals studies were conducted in accordance with the principles and procedures approved by the institutional committee for animal experiments. Five-week-old male BALB/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The animals were randomized to the following groups: (a) control (PBS); (b) celecoxib; (c) ACE inhibitor (enalapril maleate and perindopril) or ARB; and (d) celecoxib and ACE inhibitor, or celecoxib and ARB. The treatment was started 3 days before inoculation with Colon 26 cells. The cells (1×10$^4$) were injected s.c. into the shoulder region. Tumor volume was determined each week by measuring the shortest diameter and the longest diameter of implanted tumors and calculated as follows: 1/2 × (the shortest diameter) × (the longest diameter) × 0.5 (36). The mice were killed 21 days after inoculation of the cells. The tumor was removed and homogenized using a Polytron homogenizer (Kinematika AG, Littau, Switzerland) in radioimmunoprecipitation assay buffer. Centrifuged lysates were denatured, and IGF-IR expression was assessed using Western blotting procedures. IGF-IR expression levels were quantified by densitometric analysis using NIH Image Version 1.59 (NIH, Bethesda, MD).

**Immunohistochemistry.** To investigate COX-1 and COX-2 expression in Colon 26 cells \textit{in vivo}, immunohistochemical staining for COX-1 and COX-2 was performed using the avidin-biotin complex method with the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). Frozen sections were cut 5-μm thick from 3-week-old allograft tumors. Endogenous peroxidase activity was quenched by incubation in 3% H$_2$O$_2$ in methanol for 30 min. Nonspecific binding was blocked with 1.5% normal rabbit serum in PBS, and the sections were incubated with primary goat polyclonal antibodies against COX-1 (sc-1752) and COX-2 (sc-1746) [1:200 dilution; Santa Cruz Biotechnology] overnight at 4°C in 1.5% normal rabbit serum. In the negative controls, the sections were incubated in the presence of 1.5% normal rabbit serum alone overnight at 4°C. After rinsing with PBS, the sections were incubated with a biotinylated secondary rabbit antigoat antibody for 30 min and with avidin-conjugated horseradish peroxidase for 30 min. To visualize the immunohistochemical stain, the tissues were stained using 3,3'-diaminobenzidine (Vectastain DAB kit; Vector Laboratories).

**Reverse Transcription-PCR.** Total cellular RNA was isolated using Sepazole RNA 1 (Nacalai Tesque, Kyoto, Japan), and complementary DNAs were synthesized from 2 μg of total RNA using ReverTra Ace (Toyobo, Tokyo, Japan). PCR was performed in a GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems, Roissy, France) using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Cycle parameters were as follows: 1 cycle at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 1 min. Primer sets were as follows: (a) IGF-L, 5'-GCT-GAG-GTG-GAT-GCT-CTG-CTC-TTC-3' (forward) and 5'-AGT-GAT-CTT-GGC-CAT-CTG-3' (reverse); and (b) glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GCC-ATC-AAC-GCC-CCC-TTC-ATT-G-TTC-3' (forward) and 5'-ACT-CCA-CCA-CAT-ACG-CAG-C-3' (reverse).

**Cell Growth Assay.** Cells (4×10$^3$ cells/well) were plated on 96-well plates with RPMI 1640 including 10% FCS. After a 24-h incubation with 10% FCS, the cells were cultured with 0.5% FCS for 3 days. After serum starvation, the cells were treated with IGF-II for 4 days. In the groups treated with indomethacin, celecoxib, or angiotensin II, treatment was started 24 h before IGF-II treatment was initiated and continued for 5 days. The medium was changed every day. After a 4-day treatment, the net number of viable cells was determined using a water-soluble tetrazolium [2-(2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; Nacalai Tesque] colorimetric assay. The cell growth index was determined as the ratio of absorbance on day 4 to that on day 0.

**Fig. 1.** COX-1 and COX-2 expression in Colon 26 cells \textit{in vitro} and \textit{in vivo}. A, COX-1 and COX-2 expression \textit{in vitro}. COX-1 and COX-2 expression was analyzed using Western blotting procedures. B, COX-1 and COX-2 expression in allografts. COX-1 and COX-2 expression was detected by immunohistochemical staining.

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Invasion Assay. To investigate the invasion potential of cells treated with or without indomethacin, celecoxib, or angiotensin II in addition to IGF-II, an invasion assay was performed using a BD BioCoat Matrigel Invasion Chamber (Becton Dickinson, MA). Cells were preincubated with various doses of indomethacin or celecoxib with or without angiotensin II for 36 h. The cells 

\( (2 \times 10^5) \) were plated with RPMI 1640/0.5% FCS in the upper chamber containing the same dose of indomethacin or celecoxib. In the lower chamber, IGF-II (10 nM) was added to the same medium prepared as the upper chamber. After a 12-h incubation at 37°C, the cells in the upper chamber and on the upper surface of the Matrigel chamber were mechanically removed, the filters were fixed with methanol and stained with H&E, and the cells on the lower surface of the filters were manually counted.

Statistical Analysis. Statistical analyses were performed using Student’s t test and one-way ANOVA followed by Fisher’s protected least significant difference.

RESULTS

COX-1 and COX-2 Expression in Colon 26 Cells in Vitro and in Allograft in Vivo. Western blot analysis demonstrated that Colon 26 cells expressed both COX-1 and COX-2 in vitro (Fig. 1A). Immunohistochemical study demonstrated that Colon 26 allografts grown in BALB/c mice in vivo also expressed both COX-1 and COX-2 (Fig. 1B). In the allografts, both COX-1 and COX-2 were mainly expressed in Colon 26 cells. In the colon cancer cells under both in vitro and in vivo conditions, the COX-2 immunoreactivity was higher than that of COX-1.

Effect of Celecoxib, ACE inhibitors, or ARB on the Growth of Colon 26 Cells in Vitro. Treatment with celecoxib alone at a high dose (20 mg/kg) reduced tumor growth compared with the control (PBS). Treatments with celecoxib at a low dose (3 mg/kg), ACE inhibitors (enalapril maleate and perindoprilat), or ARB alone, however, did not have a significant effect against tumor growth as compared with the control. Combined treatments with celecoxib (3 or 20 mg/kg) and ACE inhibitor or celecoxib (3 or 20 mg/kg) and ARB more effective at reducing Colon 26 cell growth than the control. With respect to the dosage of celecoxib in the dual therapies, the inhibitory effects were similar between 3 and 20 mg/kg (Fig. 2). There were no differences between groups in body weight during treatment.

IGF-IR Expression in Tumors Treated with Celecoxib and Enalapril Maleate. IGF-IR expression levels in the tumors treated with celecoxib (3 mg/kg) and enalapril maleate were lower than those in tumors treated with control, celecoxib, or enalapril maleate alone (Fig. 3).

Effect of Indomethacin and Celecoxib on IGF-IR Expression. To investigate the effect of indomethacin and celecoxib on IGF-IR expression, HCA-7, LS174T, and Colon 26 cells cultured with indomethacin or celecoxib for 48 h were harvested, and IGF-IR expression levels were determined using immunoblot analysis. NSAIDs induced a dose-dependent decrease in IGF-IR expression (Fig. 4). Of the three cell lines, Colon 26 cells most strongly expressed IGF-IR. Therefore, we performed the following experiments using Colon 26 cells.

Effect of Indomethacin and Celecoxib on IGF-II-Induced Cell Growth and Invasion. IGF-II stimulation produced a 3-fold proliferation of Colon 26 cells. The maximum effective dose of IGF-II was approximately 10 nM. Cell invasion was significantly enhanced (5-fold) by IGF-II (10 nM) as compared with controls (Fig. 5A). Both indomethacin and celecoxib inhibited IGF-II-induced cell growth (Fig. 5B) and the invasion potential (Fig. 5C) in a dose-dependent manner.

Influence of PGE2/COX-2 Inhibitors on IGFs/IGF-IR Axis. Indomethacin and celecoxib did not affect the endogenous levels of IGF-I mRNA (Fig. 6A). PGE2 reversed the indomethacin and celecoxib-induced down-regulation of IGF-IR expression and the inhibitory effects on cell growth and invasion. (Fig. 6, B–D). To investigate the regulation of IGF-IR expression, we examined whether Akt activity is involved in PGE2/IGF-IR signaling. After serum-free starvation for 24 h, the cells were incubated with PGE2. Treatment with PGE2 elevated the level of phosphorylated (Ser-473) Akt (Fig. 6E, top

![Fig. 2](https://example.com/fig2.png) Effect of celecoxib, ACE inhibitors, or ARB on tumor growth in vivo. The treatment was started 3 days before inoculation of Colon 26 cells. The cells \((1 \times 10^7)\) were injected s.c. into the shoulder region of each animal using a 1-ml syringe and a 26-gauge needle. All of the drugs were delivered via daily i.p. injection. A and D, treatment with celecoxib and enalapril maleate. Mice were treated with celecoxib (3 or 20 mg/kg), enalapril maleate (10 mg/kg), or a combination of the two drugs. B and E, treatment with celecoxib and perindoprilat. Mice were treated with celecoxib (3 or 20 mg/kg), perindoprilat (2 mg/kg), or a combination of the two drugs. C and F, treatment with celecoxib and ARB. Mice were treated with celecoxib (3 or 20 mg/kg), ARB (10 mg/kg), or a combination of the two drugs. Data from each treatment group represent the means ± SD of the results in six mice. * \( P < 0.05 \) versus control (PBS).
without increasing Akt expression, and it induced IGF-IR expression. Moreover, LY294002 (10 μM) and wortmannin (100 nM) inhibited IGF-IR expression enhanced by PGE₂ treatment (Fig. 6E, bottom panel).

**Effect of Angiotensin II on IGF-IR Expression, Cell Growth, and Invasive Properties in Colon 26 Cells in the Presence of NSAIDs.** Angiotensin II reversed the indomethacin- and celecoxib-induced down-regulation of IGF-IR expression (Fig. 7A). In the absence of NSAIDs, angiotensin II did not stimulate cell growth or invasive potential (Fig. 7B). Angiotensin II partially restored growth and invasion, which were impaired by treatment with indomethacin and celecoxib, and ARB inhibited these effects of angiotensin II. PGE₂ and angiotensin II did not have synergistic effects (Fig. 7, C and D). These results suggest that angiotensin II diminished the inhibitory effect of NSAIDs on cell growth and invasion through angiotensin receptor.

In the presence of angiotensin II, treatments using NSAIDs and ARB in combination had greater effects on the inhibition of cell growth and invasion as compared with NSAIDs or ARB alone (Fig. 8, A and B). The results indicate that the inhibition of both PGE₂ and angiotensin II using NSAIDs and ACE inhibitors/ARB was more effective for the inhibition of tumor growth as compared with NSAIDs or ACE inhibitors/ARB alone.

**Fig. 3.** IGF-IR expression in tumors treated with celecoxib (3 mg/kg) and enalapril maleate. A, Western blotting analysis (top panel) and Coomassie Brilliant Blue (CBB; bottom panel). B, densitometric analysis. Representative results of each group are shown in A. Total protein (50 μg) from tumor lysates was separated on 10% SDS-polyacrylamide gels. IGF-IR expression was assessed using Western blotting procedures (top panel). The same blot was stained with Coomassie Brilliant Blue (bottom panel). B shows IGF-IR expression levels in tumors removed from 24 mice. The values of the densitometric analysis using NIH Image Version 1.59 are presented as the means ± SD of six tumors from each group and were expressed as percentages of control levels. *, *P < 0.01 versus control, celecoxib alone, and enalapril maleate alone.

**Fig. 4.** Influence of indomethacin and celecoxib on IGF-IR expression. The cells were cultured with 5% FCS including indomethacin or celecoxib for 48 h. Total protein (50 μg) from cell lysates was separated on 10% SDS-polyacrylamide gels. IGF-IR expression was assessed using Western blotting procedures. The procedures were performed three times for each cell line. The same results were obtained in all of the studies.

**Fig. 5.** Effect of indomethacin and celecoxib on IGF-II-induced cell growth and invasion. A, effect of IGF-II on cell growth and invasion. After incubation with IGF-II for 4 days, the net number of viable cells was determined using a water-soluble tetrazolium colorimetric assay. Absorbance on day 4 divided by absorbance on day 0 was defined as the growth rate. Invasion assay was performed using a BD BioCoat Matrigel Invasion Chamber. The cells (2 × 10⁴) were plated with RPMI 1640 including 0.5% FCS in the upper chamber. The lower chamber was filled with the same medium and IGF-II (10 nM) as a chemoattractant. After a 12-h incubation, the cells on the lower surface of each filter were counted. Results were expressed as number of cells/total field (X/400). The experiment was repeated three times. *, *P < 0.01 versus IGF-II (0 nm). B, influence of indomethacin and celecoxib on cell growth. Cells (4 × 10⁴) were serum-starved with 0.5% FCS for 3 days. For the last 24 h, the cells were cultured in the presence of indomethacin or celecoxib. After serum starvation, IGF-II (10 nM) was added, and the medium was changed every day for 4 days. Growth rate was calculated as described in A. *, *P < 0.01 versus control. C, influence of indomethacin and celecoxib on cell invasion. The cells were incubated with indomethacin or celecoxib for 36 h. The cells (2 × 10⁴) were plated with RPMI 1640 including 0.5% FCS and indomethacin or celecoxib in the upper chamber. The lower chamber was filled with the same medium and IGF-II (10 nm) as a chemoattractant. After a 12-h incubation, the cells invading through the filter were calculated. *, *P < 0.01 versus control.
Mechanism of Angiotensin II-Induced IGF-IR Expression. As shown in Fig. 7A, angiotensin II induced IGF-IR expression. To clarify the mechanism of angiotensin II-induced IGF-IR expression, phosphorylated (Ser-473) Akt and IGF-IR expression levels were analyzed in lysates of cells treated with angiotensin II (1 μM) using Western immunoblotting methods. Angiotensin II (1 μM) increased IGF-IR expression, and ARB inhibited the induction of IGF-IR by angiotensin II in a dose-dependent manner (Fig. 9A), indicating that angiotensin II induces IGF-IR expression through the angiotensin II receptor. Without the stimulation of angiotensin II, ARB did not influence the expression of IGF-IR as compared with the control, indicating that ARB was not cytotoxic at these doses. Moreover, angiotensin II increased phosphorylation of Akt (Fig. 9B, top panel) without increasing Akt expression, and both LY294002 (10 μM) and wortmannin (100 nM) inhibited angiotensin II-induced IGF-IR expression as well as PGE₂ (Fig. 9B, bottom panel). These results suggest that PGE₂ and angiotensin II induce IGF-IR expression through the same pathway, the PI3/Akt pathway.

DISCUSSION

IGF-IR and its ligand, IGF-II, are frequently overexpressed in human colon cancer tissues (20, 25) and have crucial roles in the growth and invasion of cancer cells (19, 21–24, 26–31). IGF-IR is
Fig. 7. Effect of angiotensin II on IGF-IR expression, cell growth, and invasive properties in Colon 26 cells in the presence of NSAIDs. A, effect of angiotensin II on IGF-IR expression in the presence of NSAIDs. Angiotensin II (1 μM) was added to cells cultured with 5% FCS including indomethacin (25 μM) or celecoxib (12.5 μM). After a 48-h incubation, IGF-IR expression was evaluated using Western blotting procedures. The procedures were performed three times. Equivalent tendencies were obtained. B, effect of angiotensin II on cell growth and invasion in the absence of NSAIDs. C, effect of angiotensin II on cell growth in the presence of NSAIDs. Cells (4 x 10^4) were serum-starved with 0.5% FCS for 3 days. For the last 24 h, the cells were cultured in the presence of indomethacin (25 μM) or celecoxib (12.5 μM) with or without angiotensin II (1 μM) and with or without ARB (20 μM). After serum starvation, angiotensin II (1 μM) and IGF-II (10 μM) were added, and the medium was changed every day for 4 days. The synergistic effect of PGE2 (0.5 μM) was also investigated. Growth rate was calculated as shown in Fig. 5A. * P < 0.01 versus IGF-II + indomethacin or celecoxib. $ P < 0.01 versus IGF-II + indomethacin or celecoxib + angiotensin II + ARB. D, effect of angiotensin II on cell invasion in the presence of NSAIDs. The cells were incubated with indomethacin (25 μM) or celecoxib (12.5 μM) with or without angiotensin II (1 μM) and with or without ARB (20 μM) for 36 h. The cells (2 x 10^5) were then plated with 0.5% FCS/RPMI 1640 with indomethacin (25 μM) or celecoxib (12.5 μM) with or without angiotensin II (1 μM) and with or without ARB (20 μM) in the upper chamber. The lower chamber was filled with the same medium and IGF-II (10 μM) as a chemoattractant. After a 12-h incubation, the number of cells invading through the filter was calculated. The synergistic effect of PGE2 (0.5 μM) was also investigated. *, P < 0.01 versus IGF-II + indomethacin or celecoxib. $, P < 0.01 versus IGF-II + indomethacin or celecoxib + angiotensin II + ARB.

The present studies demonstrate that IGF-IR is strongly expressed in all three colon cancer cell lines examined. We also confirmed that both mouse fibroblasts and Colon 26 cells expressed IGF-II (data not shown), a major ligand for IGF-IR in cancer cells, indicating that IGF-II and IGF-IR have important roles in cancer cell biology in both an autocrine and a paracrine manner. Moreover, Adachi et al. (39) reported that blockade of the IGF-II/IGF-IR axis by soluble IGF-IR inhibited growth of colon cancer xenografts in vivo. Using a hydrodynamics-based gene delivery method, we also confirmed that intrahepatic expression of soluble IGF-IR significantly inhibited liver metastasis of Colon 26 cells after intrasplenic injection.3 These results indicate that IGF-II and its receptor, IGF-IR, have a crucial role in tumor growth of colon cancer cells.

One of the mechanisms underlying the antineoplastic effects of NSAIDs is thought to be COX-2 inhibition (4, 5). COX-2 levels are elevated in 85% of colorectal carcinomas (6, 7). We demonstrated previously that COX-2 promotes the tumorigenic and metastatic potential of colorectal cancer cells (8, 9).

The results of the present study indicate that both COX-2-selective and nonselective inhibitors down-regulate IGF-IR expression in three types of colon cancer cell lines and suppress cell growth and invasion accelerated by IGF-II. We aimed to clarify the mechanism responsible for down-regulation of IGF-IR expression by COX-2 inhibition. At first, it was thought that IGF-I negatively regulates transcriptional activity of the IGF-IR gene (40). In the present study, however, 

3 Unpublished data.
indomethacin and celecoxib did not affect the level of endogenous IGF-I mRNA, indicating that indomethacin and celecoxib down-regulate IGF-IR expression through a different mechanism from the one involved with endogenous IGF-I. Secondly, COX catalyzes the conversion of arachidonic acid to PGD_{2} and PGG_{2}. PGG_{2} is subsequently converted to a variety of PGs, including PGF_{2\alpha}, PGF_{2\alpha}, PGL_{2}, and thromboxane A_{2}. We investigated the effect of PGE_{2}, a major COX-2 product, on IGF-IR expression. PGE_{2} induced IGF-IR expression in Colon 26 cells and reversed the COX-2 inhibitor-induced down-regulation of IGF-IR expression and the inhibition of cell growth and invasion. Moreover, among several possible pathways for the regulation of IGF-IR expression, Akt activation is reported to up-regulate IGF-IR expression in pancreatic carcinoma cells (33). In the present study, PGE_{2} phosphorylated Akt and PGE_{2}-induced IGF-IR expression were blocked by the PI3/Akt pathway. Sheng et al. (34) reported that PGE_{2} activates the PI3/Akt pathway in LS-174 human colorectal carcinoma cells. Our findings are consistent with this report.

Celecoxib at a high dose (>20 mg/kg) inhibited the growth of Colon 26 tumor cells, consistent with other reports (41). In the present study, the effects of celecoxib at the lowest effective dose for inhibition of PG production in vivo (3 mg/kg; Ref. 42) were investigated. Monotherapy with a selective COX-2 inhibitor at this dosage did not influence growth or IGF-IR expression in Colon 26 tumor cells in vivo. These discrepancies of the effects of COX-2 inhibitors on tumor growth and the possibly invasive properties of Colon 26 cells in vivo and in vitro were not predicted and led us to investigate other environmental factors that promote either cell proliferation or IGF-IR expression.

Angiotensin II is an endogenous factor that regulates blood pressure in humans and animals (13). Hypertension is one of the risk factors in cancer mortality (12). Furthermore, an epidemiological study indicates that long-term use of ACE inhibitors might reduce cancer mortality, whereas other antihypertensive drugs such as Ca^{2+} antagonists do not have significant antineoplastic effects (18).

Consequently, the effects of ACE inhibitors and ARB in the presence and absence of a selective COX-2 inhibitor were investigated in Colon 26 tumor cells transplanted in BALB/c mice. Colon 26 allografts in BALB/c mice have several advantages over ordinary tumor cell xenografts using athymic mice. Athymic mice lack T lymphocytes and accept tumor cells from different species. Nevertheless, athymic mice maintain certain immunological responses that probably modulate tumor cells. On the other hand, BALB/c mice maintain all immunological responses including those of T cells and B cells. Colon 26 cells originate from BALB/c mice (43). Colon 26 allografts in BALB/c mice might more accurately model tumors derived from the host's own organs. Therefore, Colon 26 cell transplants in BALB/c mice are more suitable than athymic mice models for investigating cancer cell biology in vivo. Moreover, Colon 26 cells expressed the highest levels of IGF-IR in three colon cancer cell lines overexpressing COX-2. Thus, colon 26 was the most appropriate cell line to investigate the effectiveness of the treatment on IGF-IR expression.

![Fig. 8. NSAIDs induced inhibitory effects on cell growth and invasion in the presence of angiotensin II.](image_url)

Top panel, cell growth. Cells (4 \times 10^5) were serum-starved with 0.5% FCS for 3 days. For the last 24 h, the cells were cultured in the presence of angiotensin II (1 \muM) with or without ARB (20 \muM). After serum starvation, IGF-II (10 \muM) was added to the medium, and the medium was changed every day for 4 days. B, invasion. The cells were incubated in the presence of angiotensin II (1 \muM) with indomethacin (25 \muM) or celecoxib (12.5 \muM) with or without ARB (20 \muM) for 36 h. The cells (2 \times 10^6) were then plated with 0.5% FCS/RPMI 1640 including the same medium in the upper chamber. The lower chamber was filled with the same medium and IGF-II (10 \muM) as a chemoattractant. After a 12-h incubation, the number of cells invading through the filter was counted. *, P < 0.01 versus IGF-II + angiotensin II; #, P < 0.01 versus IGF-II + angiotensin II + indomethacin or celecoxib.

![Fig. 9. Mechanism of angiotensin II on IGF-IR expression.](image_url)

A, involvement of angiotensin II receptor on the induction of IGF-IR expression by angiotensin II. The cells were serum-starved for 24 h. ARB was added 3 h before angiotensin II (1 \muM) treatment. After a 24-h incubation with angiotensin II (1 \muM), cell lysates were collected. IGF-IR expression was analyzed using Western blotting procedures. The procedures were performed three times. Equivalent tendencies were obtained. B, involvement of the PI3/Akt pathway in angiotensin II-induced IGF-IR expression. Top panel, influence of angiotensin II on Akt phosphorylation. Bottom panel, effect of PI3/Akt pathway inhibition on angiotensin II-induced IGF-IR expression. The effect of angiotensin II (1 \muM) was evaluated as described in Fig. 6E. The procedures were performed three times. Equivalent tendencies were obtained.
The present in vivo study demonstrates that monotherapy with either a COX-2 inhibitor at a low dose (3 mg/kg), an ACE inhibitor, or ARB does not suppress the growth of Colon 26 tumor cells transplanted in BALB/c mice. Dual therapy with the COX-2 inhibitor (3 or 20 mg/kg) plus ACE inhibitor/ARB, however, was effective. Furthermore, IGF-IR expression in the tumor treated with celecoxib and enalapril maleate was lower than that in the one treated with control, celecoxib, or enalapril maleate alone. With respect to the dosage of celecoxib in the dual therapies, the inhibitory effects were similar between 3 and 20 mg/kg. These results indicate that the main role of celecoxib in the dual therapies is to block the production of PGE2 because 3 mg/kg celecoxib was enough to suppress PG production. Thus, both PGE2 and angiotensin II influence IGF-IR expression and tumor growth in Colon 26 tumor cells in mice.

In the present in vitro study, angiotensin II, the target of ACE inhibitors, induced IGF-IR expression through the angiotensin II receptor and diminished the down-regulation of IGF-IR expression by a selective COX-2 inhibitor. Moreover, angiotensin II reduced the inhibitory effects of the COX-2 inhibitors on IGF-II-induced cell growth and invasion through angiotensin receptor. Indeed, in the absence of NSAIDs, angiotensin II and PGE2 did not affect IGF-II induced cell growth or invasion, but it is possible that IGF-IR expression plateaus in Colon 26 cells under basal conditions because, without NSAID treatment, additional angiotensin II or PGE2 did not further increase IGF-IR expression (data not shown).

As mentioned above, angiotensin II partially rescued cell growth and invasion inhibited by NSAIDs. Additional PGE2 treatment, however, did not have significant effects. Our results indicate that PGE2 as well as angiotensin II activated the PI3/Akt pathway, leading to the induction of IGF-IR expression, and it is possible that IGF-IR expression through PI3/Akt activation plateaus in Colon 26 cells treated with angiotensin II. Therefore, the presence of both PGE2 and angiotensin II did not synergistically induce Akt activation. Moreover, because PGE2 and angiotensin II did not completely rescue growth and invasion suppressed by NSAIDs, there are likely to be pathways other than Akt/IGF-IR by which NSAIDs inhibited cell growth and invasion and through which PGE2 cannot rescue the suppression. Many studies suggest that COX-2 products other than PGE2 are involved in proliferation and invasion (44) and that NSAIDs exercise an anti-proliferative and anti-invasive effect independently of COX-2 inhibition (45).

It is reported that angiotensin II activates Akt activity (35) and induces IGF-IR expression in vascular smooth muscle cells and Chinese hamster ovary-AT1 cells (46, 47). The present study also demonstrated that angiotensin II phosphorylated Akt and that LY294002 and wortmannin inhibited angiotensin II-induced IGF-IR expression. Therefore, angiotensin II and PGE2 regulate IGF-IR expression through the same PI3/Akt pathway. Based on our results of the in vivo and in vitro studies, we conclude that IGF-IR is a target of the combination therapies with celecoxib and ACE inhibitors or with celecoxib and ARB.

NSAIDs and ACE inhibitors might have chemopreventive effects (1–3, 18). Chemoprevention is a promising and economic strategy for cancer prevention. COX-2 inhibitors are currently used for high-risk groups such as patients with familial adenomatous polyposis in the United States. It is possible, however, that the long-term administration of COX-2 inhibitors alone at a high dose causes several side effects (10). Therefore, novel strategies are required to increase the effectiveness of COX-2 inhibitors at a low dose. Some studies indicate that new combination therapies are more effective and produce a more dramatic clinical response for the chemoprevention of human colonic neoplasia (48, 49). On the other hand, long-term use of ACE inhibitors might protect against cancer (18). ACE inhibitors are prescribed as antihypertensive agents and have few critical side effects. ACE inhibitors decrease production of angiotensin II (17), and ACE inhibitors or angiotensin II type 1 receptor antagonists are thought to affect the development of cancer (50–52). The effects of a combination therapy, however, with NSAIDs and ACE inhibitors in colon cancer have not yet been examined. This is the first report indicating that combination therapies with celecoxib at a low dose and an ACE inhibitor or with celecoxib at a low dose and an ARB can reduce tumor growth more effectively than control (PBS), celecoxib, ACE inhibitor, or ARB alone. The present in vivo and in vitro data indicate that PGE2 and angiotensin II share the same Akt/PI3 pathway, up-regulate IGF-IR expression through this intracellular signaling pathway, and promote tumor growth in colonic neoplasms. IGF-II, the ligand for IGF-IR, occurs naturally in both neoplastic and perineoplastic cells. Thus, the combination of a COX-2 inhibitor and an ACE inhibitor or of a COX-2 inhibitor and an ARB might effectively block IGF-IR-dependent tumor growth in vivo. Such combination treatments can be and possibly already are prescribed in human subjects with hypertension who require certain NSAIDs, including aspirin. Thus, the present results warrant additional epidemiological studies to clarify the effectiveness of these combination treatments as chemopreventive measures.

In conclusion, COX-2 inhibitors suppress cancer cell growth and invasion via an IGF-IR-dependent and a PI3/Akt-dependent pathway.
Angiogenesis II abolishes NSAID-induced tumor suppression via the same pathway as PGE2. Moreover, combination therapies of celecoxib and ACE inhibitor or of celecoxib and ARB reduced the amount of celecoxib needed to suppress tumor growth and lower IGF-IR expression in vivo. These results provide a possible rationale for suppressing both the COX-2/PGE2 and ACE/angiogenesis II pathways in controlling colon cancer development. Thus, combination therapies with COX-2 inhibitors and ACE inhibitors or with COX-2 inhibitors and ARB targeting IGF-IR might provide a novel and potentially promising strategy for the chemoprevention of colon cancer (Fig. 10).

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Inhibition of Angiotensin II Activity Enhanced the Antitumor Effect of Cyclooxygenase-2 Inhibitors via Insulin-Like Growth Factor I Receptor Pathway

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