Host Prostaglandin E₂-EP3 Signaling Regulates Tumor-Associated Angiogenesis and Tumor Growth

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Abstract
Nonsteroidal antiinflammatories are known to suppress incidence and progression of malignancies including colorectal cancers. However, the precise mechanism of this action remains unknown. Using prostaglandin (PG) receptor knockout mice, we have evaluated a role of PGs in tumor-associated angiogenesis and tumor growth, and identified PG receptors involved. Sarcoma-180 cells implanted in wild-type (WT) mice formed a tumor with extensive angiogenesis, which was greatly suppressed by specific inhibitors for cyclooxygenase (COX)-2 but not for COX-1. Angiogenesis in sponge implantation model, which can mimic tumor-stromal angiogenesis, was markedly suppressed in mice lacking EP3 (EP3H11002/H11002/H11002) with reduced expression of vascular endothelial growth factor (VEGF) around the sponge implants. Further, implanted tumor growth (sarcoma-180, Lewis lung carcinoma) was markedly suppressed in EP3H11002/H11002/H11002, in which tumor-associated angiogenesis was also reduced. Immunohistochemical analysis revealed that major VEGF-expressing cells in the stroma were CD3/Mac-1 double-negative fibroblasts, and that VEGF-expression in the stroma was markedly reduced in EP3H11002/H11002/H11002, compared with WT. Application of an EP3 receptor antagonist inhibited tumor growth and angiogenesis in WT, but not in EP3H11002/H11002/H11002. These results demonstrate significance of host stromal PGE₂-EP3 receptor signaling in tumor development and angiogenesis. An EP3 receptor antagonist may be a candidate of chemopreventive agents effective for malignant tumors.

Key words: angiogenesis • tumor • prostaglandin E₂ • EP3 receptor • vascular endothelial growth factor

Introduction
Nonsteroidal antiinflammatories are known to suppress incidence and progression of malignancies including colorectal cancers. However, the precise mechanism of this action remains unknown. Using prostaglandin (PG) receptor knockout mice, we have evaluated a role of PGs in tumor-associated angiogenesis and tumor growth, and identified PG receptors involved. Sarcoma-180 cells implanted in wild-type (WT) mice formed a tumor with extensive angiogenesis, which was greatly suppressed by specific inhibitors for cyclooxygenase (COX)-2 but not for COX-1. Angiogenesis in sponge implantation model, which can mimic tumor-stromal angiogenesis, was markedly suppressed in mice lacking EP3 (EP3H11002/H11002/H11002) with reduced expression of vascular endothelial growth factor (VEGF) around the sponge implants. Further, implanted tumor growth (sarcoma-180, Lewis lung carcinoma) was markedly suppressed in EP3H11002/H11002/H11002, in which tumor-associated angiogenesis was also reduced. Immunohistochemical analysis revealed that major VEGF-expressing cells in the stroma were CD3/Mac-1 double-negative fibroblasts, and that VEGF-expression in the stroma was markedly reduced in EP3H11002/H11002/H11002, compared with WT. Application of an EP3 receptor antagonist inhibited tumor growth and angiogenesis in WT, but not in EP3H11002/H11002/H11002. These results demonstrate significance of host stromal PGE₂-EP3 receptor signaling in tumor development and angiogenesis. An EP3 receptor antagonist may be a candidate of chemopreventive agents effective for malignant tumors.

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Angiogenesis is an important factor in tumor development, and tumor-associated angiogenesis is mediated by the migration and proliferation of host endothelial cells. With the use of a coculture system comprising endothelial cells and colon carcinoma cells that overexpress COX-2, Tsuji et al. (6) showed that the increased production of PGs by the carcinoma cells was associated with endothelial cell migration and tube formation, suggesting a role for COX-2 in tumor-induced angiogenesis. However, the relevance of these in vitro observations to tumorigenesis in vivo remains to be established. Furthermore, whether PGs actually contribute to the observed tumor-associated angiogenesis, and, if so, the identity of the responsible PGs and PG receptors, remains unknown.

For mechanistic analysis of angiogenesis in vivo, we have developed a sponge implantation model, in which a polyurethane sponge disk implanted subcutaneously in rats induces extensive angiogenesis in surrounding proliferative granuloma tissue (7, 8). With the use of this model, we have previously shown that angiogenesis both occurs concomitantly with the induction of COX-2 mRNA and is inhibited by administration either of a nonselective NSAID (indomethacin) or of selective COX-2 inhibitors (9). We further showed that PGE2 or the PGH2 analogue beraprost topically injected into the sponge promoted angiogenesis (9). Angiogenesis induced by either endogenous COX-2 or exogenous PGs was accompanied by increased expression of vascular endothelial growth factor (VEGF), and angiogenesis was abolished by administration of an antisense oligonucleotide specific for VEGF mRNA. These results suggest that either PGE2 or PGH2 may mediate the angiogenic action of COX-2 in situ.

PGs exert their biological actions by binding to specific receptors that contain seven transmembrane domains. Eight different PG receptors have been defined pharmacologically and cloned, including the PGD receptor (DP), four subtypes of PGE receptor (EP1, EP2, EP3, EP4), the PGF receptor (FP), the PGI receptor (IP), and the thromboxane (TX) receptor (TP) (10). Genes for each of these receptors have been disrupted and the corresponding knockout mice have been produced (11–16). Furthermore, with the use of the cloned receptors, agonists and antagonists highly selective for each of the four EP subtypes have been or are in the process of being developed (17–19).

With the use of these selective compounds and receptor knockout mice, we have now identified the PG species responsible for inducing angiogenesis in the sponge implantation model. Furthermore, we have characterized the role of PG signaling in tumor-associated angiogenesis and tumor progression in a mouse tumor implantation model. Our results indicate that host stromal PGE2–EP3 signaling appears critical for tumor-associated angiogenesis and tumor growth, and that EP3 signaling pathway is relevant to the induction of a potent proangiogenic growth factor, VEGF, which certainly has a proangiogenic action. A highly selective EP3 antagonist therefore exhibits chemopreventive action on the stromal cells, and may become a novel therapeutic tool for cancer.

Materials and Methods

Tumor Implantation. Sarcoma 180 cells, which were originally isolated from CFW mice, were cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum under a humidified atmosphere containing 5% CO2. The cells were suspended in phosphate-buffered saline at a density of 5 × 10^6 cells/ml, and 100 μl of the resulting suspension were injected into the subcutaneous tissue of male mice. Lewis Lung carcinoma cells, which were originally isolated from C57BL/6 mice, were also used in the same manner with sarcoma 180 cells in some experiments (see Fig. 3 F). One half of each specimen was assayed for hemoglobin (Hb) content, whereas the other half was embedded in paraffin, sectioned (5 μm thickness), and stained with factor VIII antibody. Microvessel density was determined following our previous report (20). All animals were housed at a constant temperature (25 ± 1°C) and humidity (60 ± 5%). All experiments were performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

Sponge Implantation Model of Angiogenesis. Sponge disks (thickness, 5 mm; diameter, 1.3 cm; references 7 and 8) were implanted under light ether anesthesia into the subcutaneous tissues of the back of 8-wk-old male ddY mice, male EP3−/− mice (14) and their wild-type counterparts, as well as IP−/− mice (11) and the corresponding WT animals. Neovascularization was assessed by the same method as described above.

Prostaglandin Levels. Fluid within the sponge matrix enclosed by granulamation tissue was gently aspirated with the use of a syringe equipped with a 25-gauge needle. The fluid was applied to a Sep-Pak C18 column, and PGs were then eluted with ethyl acetate. The eluate was dried, and the residue containing PGE2 and 6-keto-PGF1α was assayed with the use of specific ELISA (Cayman Chemical), as reported previously (21).

Immunohistochemistry. Tissue was immediately fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), dehydrated with a graded series of ethanol solutions, and embedded in paraffin. Sections (4 μm in thickness) were prepared from the paraffin-embedded tissue and mounted on glass slides; after removal of paraffin with xylene, the slides were then placed in cold (4°C) acetone. The sections were subjected to either hematoxylin–eosin staining or immunostaining. For immunostaining, the sections were first exposed to diluted normal horse serum and then incubated with either rabbit antiserum to mouse COX-2 (Cayman Chemical), rabbit antiserum to mouse VEGF (Santa Cruz Biotechnology, Inc.), rabbit antiserum to mouse Mac-1 (BD Biosciences), or rabbit antiserum to mouse CD3e (BD Biosciences). Immune complexes were detected with a Vectastain ABC kit (Vector Laboratories).

In Situ Hybridization. For in situ hybridization, dissected tissue was sectioned with a cryostat, and the resulting sections were fixed with 4% paraformaldehyde. Digoxigenin-labeled antisense and sense riboprobes for mouse EP3 mRNA were prepared by in vitro transcription of the pCRII-TOPO vector (Invitrogen) containing mouse EP3. Sections were treated with proteinase K (10 μg/ml) and were then subjected to hybridization with labeled riboprobes in hybridization solution (Novagen) for 18 h at 50°C in moistened plastic boxes. They were then exposed to RNase A (20 μg/ml) and washed extensively, and hybridized probe was detected by in-
cubation first with alkaline phosphatase–conjugated antibodies to digoxigenin and then with 5-bromo-4-chloro-3-indolyl-phosphate and 4-nitroblue tetrazolium chloride (Roche Diagnostics). The specimens were finally counterstained with hematoxylin.

RT-PCR. Transcripts encoding EP1, EP2, EP3, EP4, VEGF, CD31, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of 45 s at 94°C were amplified by RT-PCR analysis. Tissue was removed and rapidly frozen in liquid nitrogen. The frozen tissue was pulverized in a stainless steel cylinder cooled with liquid nitrogen. Total RNA was extracted from the tissue with ISOGEN (Wako), and cDNA was synthesized from 1 μg of total RNA with the use of an oligo-(dT)15 primer and AMV reverse transcriptase (Boehringer). 50 ng of cDNA were amplified with 1 U of Taq DNA polymerase in a 25 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, and 0.6 μM each of forward and reverse primers. The amplification protocol comprised 25 cycles (EP3, VEGF, CD31), 30 cycles (EP1), 40 cycles (EP2), or 40 cycles (GAPDH) of 45 s at 94°C, 60 s at 55°C, and 60 s at 72°C. The reaction mixtures were subsequently applied to a 2% agarose gel and the amplified products were stained with ethidium bromide. Primers used were as follows: 5′-GAA ATA CTA CCT CTG GTC GTG CAG CCA ACA-3′ (sense) and 5′-CCA CCA TGT ATG ATG CAG CCG GAA-3′ (antisense) for EP1, 5′-AGG ACT TCG CAG CCC CTT ACA CTTC CAA AGT-3′ (antisense) and 5′-GGA GAC ACT CAG TGA ACA AAT ATC-3′ (antisense) for EP2, 5′-GGAGGACTGTCGGACATCAGGATATC-3′ (sense) and 5′-GGAATG TTA TGA AGT CAG CCG GAA-3′ (antisense) for EP3, 5′-GGGAT CCGGATCAGGAAAA GCCAAGCCAA-3′ (sense) and 5′-CCCATATATATACAGAACACTGTTAGTGACAC-3′ (antisense) for EP4, 5′-AACATTGAA CTTTTGTGCTCT-3′ (sense) and 5′-GAG GTG TCT TGG GTC TGG CTG AGG-3′ (antisense) for CD31, 5′-GCCCTTTGCTTAGTCAGTGA AAT TTGG-3′ (antisense) for VEGF, and 5′-GGGAAGT TCTGTCAGTCTTCTAATTCGTTGTTGC-3′ (antisense) for GAPDH.

Real Time RT-PCR. Isolation of RNA and reverse transcription was performed as described above. Before transcription, all RNA samples were treated with 1 μg of DNase I (Life Technologies) per 1 μg RNA. In addition, 2.5 μg RNA from each sample was not reverse transcribed to ensure the effectiveness of the DNase treatment. Aliquots of 12.5 μg of the cDNA and gene-specific primers were added to 25 μl of qPCR Mastermix for Syber Green I (Eurogentec). Amplification reaction was performed and analyzed on ABI Prism 7700 Sequence Detector (Applied Biosystems). Primers for murine desmin were 5′-CTTC CCG CTT AGC TCT TCT CCC GTG TT-3′ (sense) and 5′-AGC TGCG AGC TTC TCC TCG TAG-3′ (antisense). Above mentioned primers for CD31 were also used. mRNA levels were normalized to β-actin mRNA and are presented as a ratio of desmin mRNA expression to CD31 mRNA.

Northern Blot Analysis. Total RNA (20 μg) extracted from tissue as described above was fractionated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde. The separated RNA molecules were transferred and covalybly bound by alkaline fixation and ultraviolet radiation–induced cross-linking to a Hybond-N+ membrane (Amershams Biosciences). For preparation of mouse VEGF and GAPDH cRNA probes, 427- and 470-bp fragments of the respective cDNAs were cloned from RAW246.7 cells and sub-cloned into the pGEM-T Easy vector (Promega). Plasmid DNA containing these fragments was linearized with NcoI or SalI, respectively, and then used as a template for synthesis of cRNA probes labeled with digoxigenin-UTP (Roche Diagnostics) by in vitro transcription with SP6 or T7 RNA polymerase. The presence of cRNA probes hybridized with mRNA on the membrane was detected with the use of alkaline phosphatase–conjugated antibodies to digoxigenin and the substrate CDP-Star (Roche Diagnostics). The membrane was exposed to x-ray film for visualization of chemiluminescence signals.

Gel Shift Assay. Primary cultured stromal fibroblasts of EP3 WT and EP3-knock out mice were stimulated with or without ONO-248 (300 nM) in DMEM containing 10% FCS for 1 h. Double-stranded oligonucleotides containing consensus sequences were labeled with digoxigenin-11-dUTP (Roche Diagnostics). The sense sequence of synthesized oligonucleotides used was 5′-GCG TTG ATG AGT CAG CCG GAA-3′. 20 μg of the nuclear extracts of fibroblasts (passage 3) were incubated with 0.155 pmol of DIG-labeled AP-1 oligonucleotide in binding buffer (10 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 5 mM DTT, 0.7 mM PMSF, 1 mg/ml poly(dI-dC)poly(dI-dC), pH 7.9) for 15 min at 25°C. For competition assay, 100-fold excess of unlabelled oligonucleotide was added to the binding reaction to the addition of the DIG-labeled probe. Then these incubation mixtures added electrophoresed in 6% native polyacrylamide gels with Tris borate-EDTA buffer. Detection of chemiluminescence was performed by using Gel Shift kit (Roche Diagnostics).

Drugs. Aspirin was provided by Merck, NS-398 (22) was obtained from Cayman Chemical, SC-560 (23) was provided by Seattle (Skokie, IL), and JTE-522 (24) was kindly supplied by Japan Tobacco (Tokyo, Japan). EP receptor selective agonists, ONO-DI-004, ONO-AE-257, ONO-AE-248, and ONO-AE-329, which were developed by us previously (18), were topically injected to the sponges. An orally active, low molecular weight inhibitor of VEGF receptor (KDR/VEGFR 2) tyrosine kinase (ZD6474) (25) was a gift from AstraZeneca (Cheshire, UK).

We newly developed an EP3 antagonist, ONO-AE3–240. The Ki values of this antagonist obtained by competition-binding isotherms to displace the radioligand binding to the respective prostanoid receptor are 590, 0.23, 58, and 1,500 nM for EP1, EP3, EP4, and FP, respectively, and more than 10 mM for EP2, DP, TP, and IP. Analysis of the agonistic and antagonistic actions showed that this compound acts as a selective antagonist at EP3 receptors. This inhibited the PGE₂ (10 nM)-induced increase in cytosolic Ca²⁺ concentration with median inhibitory concentrations of 1.5 nM for mouse EP3 receptor. This compound was dissolved in absolute ethanol at concentrations of 50 mM and 15 mM, and this stock solutions were diluted 100 times with physiological saline immediately before the topical injections. 3 d after the implantation of S-180 cells, the diluted solutions (0.1 ml/site) were subcutaneously injected around the tumors for 11 d twice a day. Physiological saline containing 1% ethanol was injected as vehicle control. An EP1 antagonist (ONO-8711; reference 17), and an EP4 antagonist (ONO-AE3–208; reference 19) were also topically injected around the tumors (twice a day, 50 nmole per tumor), in the same manner as ONO-AE3–240. Statistical Analysis. Data are expressed as means ± SEM. Comparisons among multiple groups were performed by factorial analysis of variance (ANOVA) followed by Scheffe’s test. Comparisons between two groups were performed with Student’s t test. A P value of <0.05 was considered statistically significant.

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Results

The effects of COX inhibitors on tumor growth and angiogenesis were first tested using sarcoma 180 cells, which are allogeneic for ddy mice (Fig. 1). In control ddy mice treated with vehicle, solid tumors were apparent 14 d after cell implantation. Daily oral administration of SC-560, the inhibitor selectively acting on COX-1, had no significant effect on tumor mass. In contrast, the COX-2–selective inhibitors JTE-522 and NS-398 significantly reduced tumor mass, as did aspirin, a nonselective COX inhibitor (Fig. 1, A and D). The extent of tumor-induced angiogenesis was assessed on the basis of hemoglobin contents (Fig. 1 C), which correlated well with the vascular density in the tumor under histological examination (Fig. 1 B). Consistent with the marked red color of the tumors, angiogenesis was substantial in vehicle-treated mice (Fig. 1, B and C). Similar to the findings in tumor mass, angiogenesis was greatly reduced by treatment with COX-2 inhibitors or aspirin, but not with SC-560 (Fig. 1, B and C). These results suggested that COX-2 was involved in tumor growth and angiogenesis also in this model.

To test whether PGs generated by COX-2 are involved in angiogenesis and, if so, to identify a PG species and a PG receptor involved, we next employed a sponge implantation model that we developed previously. This model utilizes a polyurethane sponge implanted subcutaneously in mice, which induces proliferative granulation tissue around the implant and extensive angiogenesis within this encapsulation in a COX-2–dependent manner, thus mimicking the stromal angiogenic response around tumors. In the present experiment, to identify the receptor mediating the above action, we topically injected recently developed EP agonists that are highly selective for each subtype. Neither the EP1 agonist ONO-DI-004, nor the EP2 agonist ONO-AEI-257, nor the EP4 agonist ONO-AEI-329 enhanced significant angiogenesis (Fig. 2 A). In contrast, the EP3 agonist ONO-AE-248 markedly increased the extent of angiogenesis in a dose-dependent manner (Fig. 2 A). Administration of ONO-AE-248 significantly increased the rate of angiogenesis, with the maximal effect at day 14 (Fig. 2 C). These were also true in the mice treated with a COX-2 selective inhibitor, JTE-522 (Fig. 2 B). These results suggest a role of the PGE$_2$-EP3 receptor signaling in the sponge-induced angiogenesis.

To verify the role of endogenous PGE$_2$, we applied the sponge model to mice deficient in EP3 receptor (EP3$^-/-$). We also used mice deficient in IP receptor (IP$^-/-$), because endogenous PGI$_2$ levels were increased in sponge implantation models. Their respective WT counterparts, were used as controls. The extent of angiogenesis in EP3$^-/-$ mice was significantly reduced compared with that in WT mice (Fig. 2 D). In contrast, angiogenesis was significantly enhanced in IP$^-/-$ mice compared with that in WT animals (unpublished data). The impaired angiogenic response in EP3$^-/-$ mice was due to loss of receptors and not due to a difference in the production of PGs, as the amounts of PGE$_2$ and 6-keto-PGF$_{1alpha}$, a stable metabolite of PGI$_2$, in the sponge implants in EP3$^-/-$ mice (345 ± 67 and 299 ± 28 pg per sponge, n = 6, respectively) were not significantly different from those in WT mice (260 ± 46 and 255 ± 39 pg per sponge, n = 6, respectively). The expressions of VEGF in sponge granula-
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tion tissues were markedly reduced in EP3−/− mice, compared with those in WT mice (Fig. 2, E and F). These results taken together demonstrate a significant role of endogenous PGE2 in the sponge-induced angiogenesis, and suggest that EP3 receptor mediates this proangiogenic action of PGs. EP3 signaling is a major pathway for this angiogenesis, as topical injections of either EP3 agonist or PGE2 did not enhance angiogenesis at all in EP3−/− mice (data not shown). Expressed VEGF certainly enhanced angiogenesis in this model, as VEGF antibody significantly reduced angiogenesis compared with control IgG (Fig. 2, G and H).

We then investigated whether the PGE2-EP3 signaling also operates in tumor-induced angiogenesis by comparing tumor growth and angiogenesis in four kinds of EP receptor knockout mice, IP−/−, and their WT counterparts injected with sarcoma 180 cells. Sarcoma 180 cells are allogeneic for the prostanoid receptor knockout mice, which was developed from C57BL/6 mice. Solid tumors that formed in EP3−/− mice were significantly smaller than those in WT mice (Fig. 3, B and D). Indeed, 14 d after the implantation of tumor cells, both tumor mass and angiogenesis, the latter assessed from microvessel density and hemoglobin...
content, were significantly reduced in EP3−/− mice compared with those in WT mice (Fig. 3 A–C). The extent of reduction in angiogenesis in EP3−/− (by 76%) was certainly larger than that observed in EP2−/− (by 46%), although angiogenesis in EP2−/− mice was also significantly reduced when assessed by microvessel density (Fig. 3 A). Even in the early stage of the experiment (7 d after implantation), the reduced angiogenesis and tumor growth were observed (Fig. 3, A–C). Fig. 3 D shows typical appearance of tumors formed in EP3−/− and WT mice. Histological examination of tumors formed in WT mice revealed an extensive vasculature in the capsule where tumors mingled with the surrounding normal tissue. In contrast, the tumors formed in EP3−/− mice exhibited both a low level of vascularization and distinct boundaries with the surrounding normal tissue (Fig. 3 E). We observed a difference of tumor growth between EP3−/− mice and WT mice using another neoplastic cell line, Lewis Lung carcinoma, which are syngeneic for C57BL/6 mice (Fig. 3 F). These results suggest that the EP3 receptor in the host has a major and critical role in tumor-induced angiogenesis and tumor growth. As shown below (see Fig. 5 A), the major EP receptor expressed in subcutaneous tissues in WT mice was EP3.

To corroborate the interaction between tumors (sarcoma 180) and the surrounding stroma, we examined COX-2 and VEGF expression in tumor and stromal tissue by immunohistochemical analysis in allogeneic models. Intense staining for COX-2 was apparent in sarcoma cells of tumors that formed in WT mice; less marked staining was also apparent in the stromal cells surrounding the tumors in these animals (Fig. 4 A, left). Substantial COX-2 immunoreactivity was also detected in the tumors together with stromal cells that formed in EP3−/− mice, although the extent of neovascularization in the capsular stroma was markedly reduced (Fig. 4 A, right). In WT mice, VEGF was abundant in surrounding stromal cells (Fig. 4 B, left), which major components were Mac-1 and CD3 double-negative fibroblast-like cells (Fig. 4, F and G), and was also present in smaller amounts in tumor cells (Fig. 4 B, left). The number of stromal cells, as well as of tumor cells, expressing VEGF was markedly reduced in EP3−/− mice (Fig. 4 B, right). In situ hybridization revealed that EP3 mRNA was localized in both sarcoma and stromal cells (Fig. 4 C), but not in endothelial cells (Fig. 4 D, arrowhead), in WT mice, but was apparent only in the sarcoma cells in EP3−/− mice (unpublished data).

RT-PCR analysis confirmed these findings on expression of VEGF and EP3 mRNA in tumor (sarcoma 180) and capsular stromal tissue (T+S)t from WT mice and EP3−/− mice. The expressed levels of other PGE receptor subtypes, EP1, EP2, and EP4, in both T+St and control subcutaneous tissue (H) were not apparently different between EP3−/− mice and WT mice (Fig. 5 A). It further detected transcripts encoding CD31 in the tissue from WT mice but not...
in that from EP3−/− mice (Fig. 5 A). The ratio of the expression of desmin mRNA to that of CD31 mRNA determined in the samples isolated from the normal subcutaneous tissue (H) was not different between EP3−/− mice and WT mice (Fig. 5 B). But, in the samples of sarcoma 180 tumors including surrounding stroma (T+St), which were prepared in the same manner as PCR analysis experiment (Fig. 5 A), the ratio was reduced in both EP3−/− mice and WT mice, compared with that in the normal subcutaneous tissues isolated from each mouse (Fig. 5 B). Further, the reduction was more significant in EP3−/− mice in comparison with that in WT mice (Fig. 5 B).

Daily topical injections of VEGF antibody significantly reduced tumor growth and angiogenesis in WT mice, but not in EP3−/− mice, suggesting that VEGF is a predominant factor to induce tumor growth and angiogenesis in this model in response to EP3 signaling (Fig. 4 J). The lack of inhibition in tumor-associated angiogenesis in EP3−/− mice was also observed in the mice treated with the inhibitor of tyrosine kinase of VEGF receptor, which was effective in WT mice (Fig. 5 E).

Gel shift assays of several transcriptional factors (AP-1, AP-2, SP-1, HIF-1, NF-κB, and Oct-2A) in fibroblasts isolated from the stroma in EP3−/− mice and WT mice, revealed that of the factors tested, AP-1 activation was significantly reduced in EP3−/− mice, compared with WT mice, after stimulation with a selective agonist for EP3, ONO-AE-248 (Fig. 5 C). AP-1–dependent up-regulation of VEGF may be important in tumor-associated angiogenesis mediated by EP3 receptors.

Recently, we succeeded to develop a potent and selective EP3 antagonist, ONO-AE3–240. Daily topical injections of this EP3 antagonist around the tumor (sarcoma 180) significantly suppressed tumor-associated angiogenesis and tumor growth in WT mice, whereas those of an EP1 antagonist and an EP4 antagonist did not (Fig. 6 A). This chemopreventive effect of an EP3 antagonist was not seen in EP3−/− mice (Fig. 6 B).
Expression of VEGF, EP receptor subtypes, and CD31 in tumors and surrounding stroma tissues in C57BL/6 WT mice and EP3 knockout mice, and effects of a VEGF neutralizing antibody and a VEGF receptor kinase inhibitor on tumor growth and tumor-associated angiogenesis.

Sarcoma 180 cells, which were allogeneic for C57BL/6 mice, were injected into the subcutaneous tissue. (A) RT-PCR analysis was performed as described in the methods, and a ratio of desmin mRNA/CD31 mRNA in tumor tissue dissected together with surrounding stroma (T + ST), as well as in control subcutaneous tissue (H) dissected from sites distant from tumors, of WT and EP3<sup>-/-</sup> mice. 14 d after implantation, sample tissues were isolated. (B) A ratio of desmin mRNA/CD31 mRNA. Real-time RT-PCR analysis was performed as described in the methods, and a ratio of the expression of desmin mRNA to that of CD31 mRNA was determined. Data are means ± SEM for 4 samples prepared as above. *P < 0.05 compared with H, and #P < 0.05 compared with T + ST in EP3WT (ANOVA). (C) Gel shift assay. Primary cultured fibroblasts from WT and EP3<sup>-/-</sup> mice were stimulated with ONO-AE-248, a EP3 agonist. Activation of AP-1 was determined by EMSA. Lanes 1, 2, and 7, or 3 to 6, without or with ONO-AE-248; lanes 4 and 6, presence of unlabeled oligonucleotide with nuclear extract, by which specificity was evaluated by oligonucleotide competition and EMSA; lane 7, control oligonucleotide without nuclear extract. (D) and (E) Effects of an anti-VEGF antibody and a VEGF receptor kinase inhibitor on tumor growth and angiogenesis in WT and EP3<sup>-/-</sup> mice. Tumor-associated angiogenesis was determined 14 d after injection of sarcoma 180 cells. Either IgG specific for mouse VEGF (10 μg per tumor per day, once a day) or nonimmune control IgG was topically injected around the tumors (D). Either VEGF receptor kinase inhibitor, ZD6474 (100 mg/kg, once a day) or vehicle control solution was orally administered (E). Data are means ± SEM for 4 samples prepared as above. *P < 0.05 compared with WT mice receiving control IgG or vehicle solution (ANOVA).

To mimic stromal angiogenic responses, we developed the sponge implantation model. Two advantages of this model for studies of angiogenesis are that angiogenesis can be readily quantified by measurement of the hemoglobin content of the sponge-induced granulation tissue together with histological examination, and that the effects of exogenous substances can be investigated by their direct injection into the sponge (7–9). The EP3 agonist ONO-AE-248 specifically enhanced angiogenesis in this model in a dose-dependent manner, and further the angiogenic response was certainly reduced in EP3<sup>-/-</sup> with the reduction of VEGF expression, suggesting that endogenous PGE<sub>2</sub> facilitates angiogenesis through the EP3 signaling and the induction of VEGF in this model. Previous studies with various cell lines have indicated that PGE<sub>2</sub> induces VEGF expression through a cAMP-dependent mechanism (34, 35). We previously reported that PGE<sub>2</sub> induces VEGF through the activation of adenylate cyclase/protein kinase A signaling pathway (36). One of the EP3 splicing variants (37, 38), which may couple to the elevation in intracellular cAMP levels, may enhance angiogenesis in this model.

It has been previously reported that E type PGs have a proangiogenic activity in corneal test (39) and in the chorioallantoic membrane (CAM) technique (40). Further, Form and Auerbach reported that PGE<sub>2</sub> strongly induced angiogenesis on CAM of 8-d-old chicken embryos, but PGA<sub>2</sub> and TXA<sub>2</sub> antagonist, the former of which inhibited more strongly than the latter, suggesting the involvement of EP3.<ref>228 EP3 Receptor and Tumor Growth</ref>
other COX-2 products than TXA₂. However, these in vivo results were obtained under bFGF-stimulated conditions. In our separate experiment, angiogenesis in the sponge implantation model under no stimulation was not reduced with either a thromboxane synthase inhibitor, OKY046 or a TP receptor antagonist, S-1452 (unpublished data). The contribution of TP receptor signaling to tumor-associated angiogenesis should be estimated carefully. In the present experiments, we tested tumor-associated angiogenesis in knockout mice of EP subtypes or IP receptors (Fig. 3, A and C). As shown in the present study, not only tumor growth but also tumor-associated angiogenesis are highly dependent on EP3 receptor signaling. Judging from the time course of changes in size and angiogenesis of tumors in EP3⁻/⁻ and WT mice (Fig. 3, A–C), the reduced tumor growth and angiogenesis in EP3⁻/⁻ mice is not a simply minor delay. However, angiogenesis and growth of polyps in mice with a mutated APC gene were recently reported to be EP2-dependent (42, 43). Their report described that the major elements which express COX-2 are stromal cells around the tumors or intestinal polyps. We identified that the cells which produced VEGF to facilitate angiogenesis and tumor growth were CD3 and Mac-1 double-negative fibroblasts. It is widely known that the fibroblasts exhibit heterogeneity in term of various biological factors including prostaglandin generating systems and receptor systems (44–46). We have shown in the present study, not only tumor growth but also tumor-associated angiogenesis are highly EP3 dependent. They did not show the microvessels density in EP3⁻/⁻ with APC mutation in their recent report (43), and the reduction percentage of angiogenesis in APC-mutated EP2⁻/⁻ mice was ~30% at best. As shown in Fig. 5 A, the major EP receptor expressed in subcutaneous tissues in WT mice was EP3, which did not expressed in the intestine (43). These suggested that tumor-associated angiogenesis may be regulated in a site-specific fashion, and may be related to the heterogeneity of the stromal fibroblasts.

The mouse strains, ddy mouse and C57BL/6 mouse are allogeneic for S-180 sarcoma cell line used in the present study. However, we tested another cell line, Lewis lung carcinoma, for which C57BL/6 mouse is syngeneic (Fig. 3 F). The difference of tumor growth in EP3⁻/⁻ mice and WT counterparts was observable in both tumor cell lines. These suggested that EP3 receptor signaling to facilitate the tumor growth was important not only in allogeneic tumors but also in syngeneic tumors.

The host microenvironment is thought to influence tumor progression (47, 48). Examination of human colorectal cancer tissue has revealed marked COX-2 expression not only in cancer cells but also in inflammatory cells and fibroblasts that surround the cancer cells (34). Ohshima et al. (2) also showed that COX-2 is abundant in the stromal cells that surround intestinal polyps in mice with a mutated APC gene. Recent results using COX-2 knockout mice also supported the significance of stromal COX-2 in tumor-induced angiogenesis and tumor growth (49). In the present study, we have focused on the downstream signal-
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Expression was also reported in several kinds of samples of S-180 tumors including surrounding stroma (T+ST), the ratio was reduced in both EP3^+/− mice and WT mice, compared with that in the normal stromata isolated from each mouse. Further, the reduction was more significant in EP3^−/− mice in comparison with that in WT mice. These results taken together suggest that the development of pericytes on the newly formed vessels in association to tumors is EP3 receptor signaling-dependent, and that the equipment of the pericytes in the vessels (arterioles and venules) in normal tissues is not.

Among the various factors, VEGF may be a candidate to enhance angiogenesis in sponge model judging from the results using VEGF neutralizing antibody (Fig. 2, G and H). Further, daily topical injections of VEGF antibody significantly suppressed tumor-associated angiogenesis and tumor growth in WT mice, whereas those of an EP1 antagonist and an EP4 antagonist did not (Fig. 6 A). This chemopreventive effect of an EP3 antagonist was not seen in EP3^−/− mice (Fig. 6 B). These results certainly confirm the results from EP3^−/− mice.

In conclusion, as shown in Fig. 6 C, host stromal PGE2–EP3 signaling appears critical for tumor-associated angiogenesis and tumor growth. EP3 signaling on the stromal cells was relevant to the induction of a potent proangiogenic growth factor, VEGF in stromal cells. Up-regulated VEGF certainly has a proangiogenic action, and facilitates tumor growth. A highly selective EP3 antagonist therefore exhibits chemopreventive action on the stromal cells, and will become a novel therapeutic tool for cancer. The results presented here neatly provide an answer to the question how NSAIDs prevent tumor development.

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