Significant Roles of Inducible Cyclooxygenase (COX)-2 in Angiogenesis in Rat Sponge Implants

Masataka Majima[1], Masako Isono[1], Yasuhiro Ikeda[1], Izumi Hayashi[1], Ko Hatanaka[1], Yoshiteru Harada[1], Osamu Katsumata[2], Shohei Yamashina[2], Makoto Katori[1] and Shozo Yamamoto[3]

[1]*Department of Pharmacology and* [2]*Department of Anatomy, Kitasato University School of Medicine, Kanagawa 228, Japan*
[3]*Department of Biochemistry, Tokushima University School of Medicine, Tokushima 770, Japan*

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ABSTRACT—Angiogenesis in rat sponge implants, as determined from the concentration of hemoglobin in the sponge granuloma tissues, was gradually increased over a 14-day experimental period. The inducible cyclooxygenase COX-2 was detected in the sponge granuloma tissues at day 4 by Western blot analysis using specific mouse COX-2 antibody. Angiogenesis in the sponge implants was enhanced by daily topical injections of human recombinant basic fibroblast growth factor (bFGF) or human recombinant epidermal growth factor (EGF) (100 or 1000 ng/sponge/day) for 4 days. These treatments clearly enhanced the expression of COX-2 in the sponge granuloma tissues. In immunohistochemical studies, COX-2-positive staining was mainly observed in the endothelial cells of the neovasculature and in the fibroblasts of the granuloma capsule. Administration of the selective COX-2 inhibitor NS-398 (p.o., 3 mg/kg, 3 times a day) for 14 days significantly inhibited the angiogenesis. The angiogenesis enhanced with bFGF or EGF (day 4) was inhibited by administration of indomethacin or NS-398, both in the above regimen, and fell to the level obtained without growth factor treatment. These results suggest that COX-2 induced in the sponge granuloma tissues may participate in neovascularization through prostaglandin formation.

Keywords: Angiogenesis, Cyclooxygenase (COX)-2, Sponge implant, Growth factor, Immunohistochemistry

Angiogenesis can be defined as the development of new blood vessels from an existing vascular bed. Normal vascular proliferation occurs only during embryonic development, the female reproductive cycle and wound healing. By contrast, many pathological conditions such as cancer, rheumatoid arthritis, atherosclerosis and diabetic retinopathies are characterized by persistent, unregulated angiogenesis. Thus, an effective strategy for anti-angiogenesis may provide a novel therapeutic approach for the treatment of such diseases (1－4). In fact, since the first successful clinical treatment of pulmonary hemangioendotheliomas with interferon α-2a, several anti-angiogenic agents are currently being evaluated for their efficacy as anti-cancer drugs (5).

E-type prostaglandins, such as prostaglandin E_1 and prostaglandin E_2, were reported to enhance the angiogenesis in vivo (6, 7). The generation of prostaglandin was generally regulated by the several enzyme activities. Cyclooxygenase (COX) is one of the most important enzymes involved in prostaglandin formation. The rate of generation may depend on the activity of the COXs in physiological or pathological conditions. Since COX inhibitors inhibit angiogenesis in the cornea (6, 8), which is enhanced by topical administration of E-type prostaglandins (6, 9), the prostaglandins generated in the cornea may enhance the angiogenesis in this model. Recently, two isoforms of COX have been identified (10, 11). Whereas COX-1 is expressed constitutively in various types of cells, the expression of COX-2 is induced by various stimuli (11). We previously reported that COX-2 was selectively expressed in the endometrium, whose thickness was increased during estrus (12).

In the present study, we detected the expression of COX-2 in the granuloma tissues of sponge implants in rats. In addition, we tested the effect of a selective COX-2 inhibitor on the angiogenesis in the sponge implants to reveal the roles of the expressed COX-2.

MATERIALS AND METHODS

The rat sponge model

Circular sponge discs (5-mm-thick, 1.3 cm in diameter, and weighing 14.2±0.1 mg, n=5) were prepared from a sheet of polyether ployurethane foam by use of a wad pouch (13). The discs were soaked in 70% ethanol for 3 hr and then rinsed in sterile distilled water. After drying at reduced pressure, all discs were exposed to ethylene oxide gas. Under light ether anesthesia, they were then implanted into the subcutaneous tissue of the backs of male Sprague-Dawley strain rats (specific pathogen-free, 8-weeks-old), purchased from SLC (Hamamatsu). All rats were kept at constant temperature (25±1℃) and humidity (60±5%) with free access to normal chow and water throughout the experimental periods.

Neovascularization was assessed by measuring the concentration of hemoglobin in the granuloma tissues together with the enclosed sponge implants (14). Briefly, the granuloma tissues with sponge were taken immediately after the death induced by the intraperitoneal administration of excess doses of sodium pentobarbital. After the granuloma tissues including sponge were weighed, they were cut into several pieces with scissors. Distilled water 4 times the weight of the sample granuloma tissues was added to each sample, which was then homogenized with a Polytrone homogenizer (Kinematica GmbH, Luzern, Switzerland). The hemoglobin concentration in the supernatant after centrifugation at 5,000×*g* was determined by a hemoglobin assay kit (Hemoglobin B Testwako; Wako Pure Chemical Industries Ltd., Osaka). The concentrations of hemoglobin in the granuloma tissues were expressed as mg/g wet tissue.

To enhance the neovascularization of the sponge implants, basic fibroblast growth factor (bFGF, 100 or 1000 ng/sponge/day), epidermal growth factor (EGF, 1000 ng/sponge/day), or their vehicle solution (0.5% BSA, 0.1% CHAPS in physiological saline) were daily injected (once a day, 0.05 ml/sponge) into the sponge implants using a 26G needle under light ether anesthesia.

Western blot analysis of COX isozymes

The granuloma tissues were removed from the rats and then washed with phosphate-buffered saline. The tissues were cut with scissors into small pieces in 100 mM Tris-HCl buffer (pH 7.4) containing 5 mM tryptophan and homogenized with a Polytron. After sedimentation of the cell debris by centrifugation at 12,000×*g* for 1 hr at 4℃, the homogenates were centrifuged at 100,000×*g* for 1 hr at 4℃. This microsomal fraction pellet was then solubilized in 0.5% Tween 20.

For immunoblot analysis of COX isoenzymes, solubilized protein (50 to 75 μg/lane) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to an Immunobilon-P membrane (Millipore Japan, Tokyo) with the use of semidry blotting (12). After blocking with Block Ace (Dainippon Pharmaceutical Corp., Osaka), the blot membrane was incubated with rabbit anti-bovine COX-1 anti-serum, which was used in our previous study (12) or with rabbit anti-murine-COX-2 anti-serum (Cayman Chemical, Ann Arbor, MI, USA) (12). The membrane was then incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase and stained with Konica Immunostain (Konica, Tokyo) (12). Molecular marker protein was purchased from Pharmacia LKB (Uppsala, Sweden).

Immunohistochemical studies

Immunohistochemical studies were performed as described in our previous report (15).

Briefly, the granuloma tissues were immediately fixed with 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4). After fixation, the tissues were dehydrated with a graded series of ethanol solutions and embedded in paraffin. The sections (3 μm) from the paraffin-embedded tissues were mounted on glass slides, deparaffinized with xylene, and then placed in cold (4℃) acetone for immunostaining.

The procedure for staining dehydrated sections using a Vectastain ABC Kit (Vector Lab., Burlingame, CA, USA) was as follows: 1) incubation with diluted normal horse serum; 2) incubation with diluted (×200) rabbit anti-murine-COX-2 anti-serum (Cayman Chemical); 3) incubation with biotinylated anti-rabbit IgG (7 mg/ml); 4) incubation with avidin-biotin-peroxidase complex; 5) placement in 0.02% 3,3′-diaminobenzidine (DAB) and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.4); 6) color development by immersion in the DAB solution containing 0.005% H_2O_2; 7) examination and photography with a light microscope.

Histology

The sample sponges were removed from the backs of the rats and were then immediately fixed with 10% formalin solution at 4℃. Sections (5 μm) were prepared and stained with hematoxylin and eosin or van Gieson's method (16).

Administration of COX inhibitors

To the rats receiving bFGF (100 or 1000 ng/sponge /day), EGF (1000 ng/sponge/day) or to the rats that had no growth factor treatment, COX inhibitors were administered orally. From the first day of the implantation, indomethacin in a suspension of 3 mg/ml in 5% gum arabic in distilled water was given 3 times a day (every 8 hr) at the dose of 3 mg/kg. A selective COX-2

inhibitor, NS-398 (17), was also administered, as a suspension of 3 or 30 mg/ml in 5% gum arabic in distilled water, either twice (30 mg/kg/12 hr) or three times (3 mg/kg/8 hr) a day during the experiments. For the vehicle control rats, only vehicle solution (5% gum arabic in distilled water) was administered (1 ml/kg) orally twice or 3 times a day.

In a separate experiment, NS-398 was administered (3 mg/kg/8 hr) orally only for 2 days to one group of rats (days 1 and 2 of the experiment), and then vehicle solution was administered for 2 days (days 3 and 4). To another group of rats, NS-398 was administered for 2 days (days 3 and 4), following 2 days vehicle treatment (days 1 and 2). In both groups of rats, bFGF (1000 ng/site) was administered topically to the implanted sponges for 4 days.

Chemicals and other materials

EGF and bFGF was purchased from Genzyme Corp. (Cambridge, MA, USA). Indomethacin was a gift from Merck (Rahway, NJ, USA), and NS-398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide) was also a gift from Taisho Pharmaceutical Corporation (Tokyo). All other substances used were of analytical grade and were purchased from commercial sources.

Statistical analyses

Values were expressed as means ± S.E.M.

The unpaired Student's *t*-test was used to evaluate the significance of differences between the results of two groups, when variances were not heterogeneous; and when they were heterogeneous, statistical analysis was performed by the Aspin-Welch method or by Wilcoxon's rank sum test.

For the results from multiple groups, factorial ANOVA was used, followed by Scheffe's test.

A P value of less than 0.05 was considered to be significant.

RESULTS

Changes in the weights of sponge granuloma tissues

As Table 1 shows, the weights of sponge granuloma tissues without the topical injections of growth factor were gradually increased over a 14-day experimental period. Daily injections of EGF (1000 ng/site) for 4 days into the sponge implants for 4 days caused a slight increase in the weights of the granuloma tissues, compared with those in vehicle-treated rats, although the difference was not statistically significant. Daily injections of bFGF at doses of 100 and 1000 ng/site for 4 days significantly increased the weights of the granuloma tissues in a dose-dependent manner.

Table 1. Changes in weight (mg) of sponge granuloma after subcutaneous implantation

Treatment	Days after implantation		
	4	9	14
Vehicle	434 ± 24	569 ± 45*	1329 ± 132*
EGF (1000)	510 ± 55	—	—
bFGF (100)	671 ± 109*	—	—
bFGF (1000)	900 ± 53*	—	—

Each value represents the mean ± S.E.M. from 5–8 animals. Epidermal growth factor, EGF; basic fibroblast growth factor, bFGF; EGF (1000), EGF (1000 ng/site/day); bFGF (100), bFGF (100 ng/site/day); bFGF (1000), bFGF (1000 ng/site/day). *$P < 0.05$, comparison with the value from vehicle-treated rats on day 4.

Histology

The connective tissues around the implanted sponges (day 4), which were constituted mainly of collagen fibers, appeared on van Gieson staining as reddish fibers (Fig. 1: A–D). As shown in Fig. 1, C and D, when a higher dose of bFGF (1000 ng/site) was injected topically for 4 days, the implanted sponge was encapsulated with thick fibrous connective tissue. The capsules that formed around the sponge implants were certainly thicker than those that formed without the injection of growth factor (Fig. 1: A and B). Newly developed capillaries and more extensive vascularization were evident in areas of bFGF-stimulated fibrous encapsulation. Infiltration of mononuclear cells accompanied by a few polymorphonuclear cells was observed in the area of encapsulation. These infiltrated cells were also predominant in bFGF-treated sponges (Fig. 1: C and D).

Time course of angiogenesis in sponge granuloma tissues

As shown in Fig. 2, angiogenesis without growth factor injection gradually increased over a 14-day period, as assessed from the hemoglobin concentration of the sponge granuloma tissues. Daily injections of EGF (1000 ng/site) into the sponge implants caused a marked increase in angiogenesis from the second day of administration. In the case of bFGF also, daily injections for 4 days increased the angiogenesis in a dose-dependent manner. Hemoglobin concentrations of sponge implants receiving 1000 ng/site of bFGF for 4 days increased to almost the same levels as those without bFGF on day 14.

Detection of inducible COX-2 in sponge granuloma by Western blot analysis

As shown in Fig. 3A, in the granuloma tissues of the sponge implants without growth factor stimulation, constitutive COX-1 was detected throughout the experimental periods. Even with the stimulation of EGF (1000

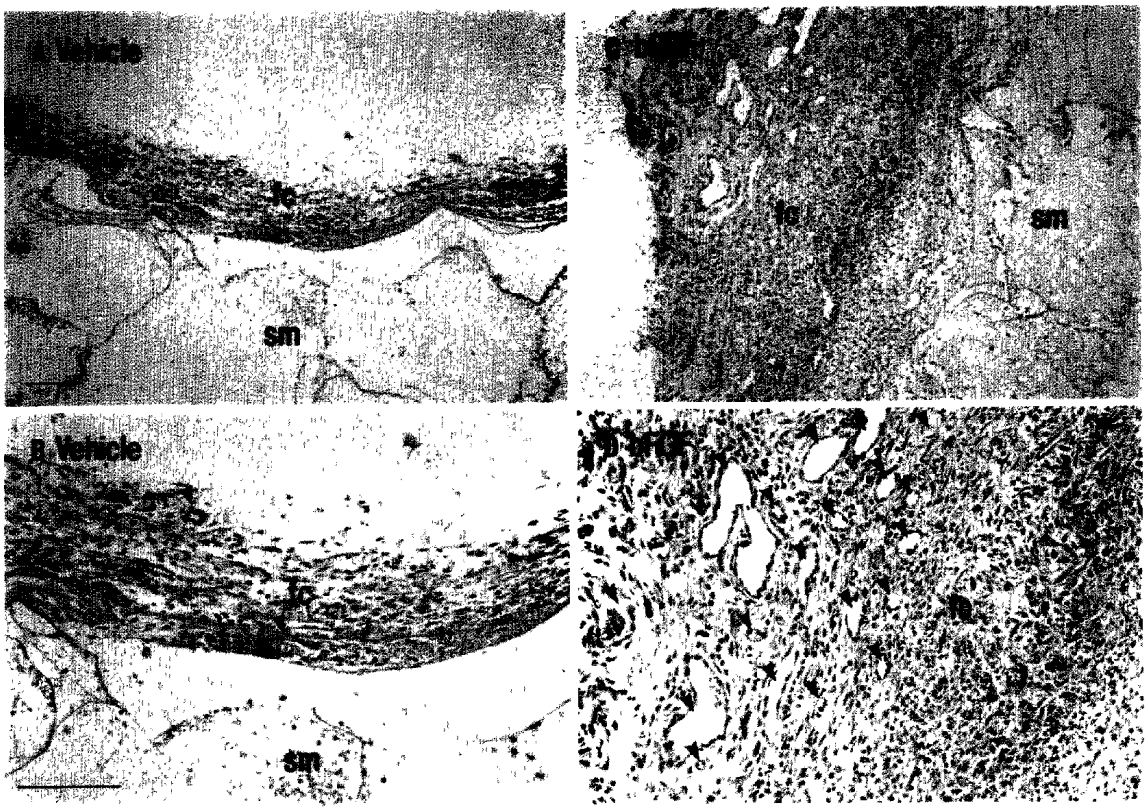


Fig. 1. Histological sections of the sponge granuloma at day 4 with and without bFGF injections (1000 ng/site/day). Sections were stained by the van Gieson method. Each bar represents 50 μm. Arrowheads indicate newly developed blood vessels. fc, fibroblastic capsule; sm, sponge matrix. High magnification: Panels B and D.

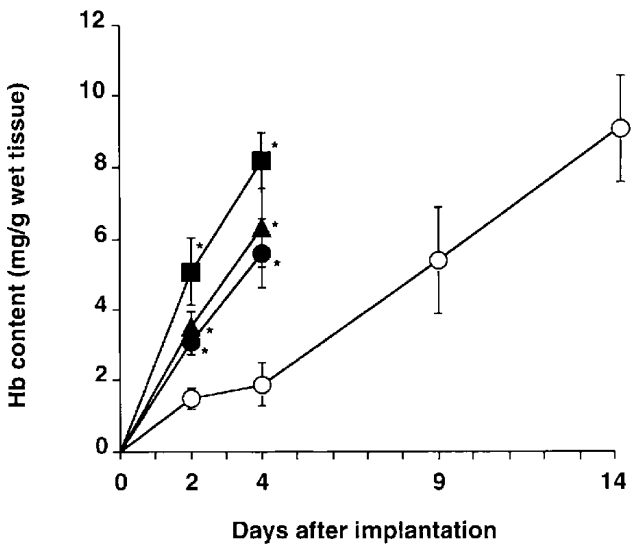


Fig. 2. Changes in hemoglobin concentration in sponge granuloma with and without growth factor stimulation. Substances injected into the sponge implants once a day: basic fibroblast growth factor (●, 100 ng/site/day; ■, 1000 ng/site/day); epidermal growth factor (▲, 1000 ng/site/day); or vehicle solution (○). Each value represents the mean ± S.E.M. 5 to 8 animals. *$P < 0.05$ vs vehicle control.

ng/site) or bFGF (100 and 1000 ng/site) for 4 days, COX-1 was detected; its detected levels were not affected by growth factor treatment (Fig. 4A).

In contrast, the inducible COX-2 was detected only on day 4 in the sponge granuloma tissues without growth factor treatment (Fig. 3B). After day 4, COX-2 was not detected under the same experimental condition (Fig. 3B). On day 4, the detected levels of COX-2 in the sponges were markedly enhanced by a 4-day treatment with EGF (1000 ng/site) or bFGF (100 and 1000 ng/site) (Fig. 4B).

Immunohistochemical localization of inducible COX-2 in sponge granuloma

In the immunohistochemical studies, the COX-2 positive stains were found mainly on the endothelial cells of vessels that developed newly in the fibrous capsules of vehicle-treated (Fig. 5A) and bFGF-treated (Fig. 5B) sponge granuloma. The predominant positive reactions were those in the sponge implants receiving bFGF stimulation (Fig. 5B). Weak positive reactions were also observed on the fibroblast-like cells located around the newly-formed vessels. No marked positive staining was observed on

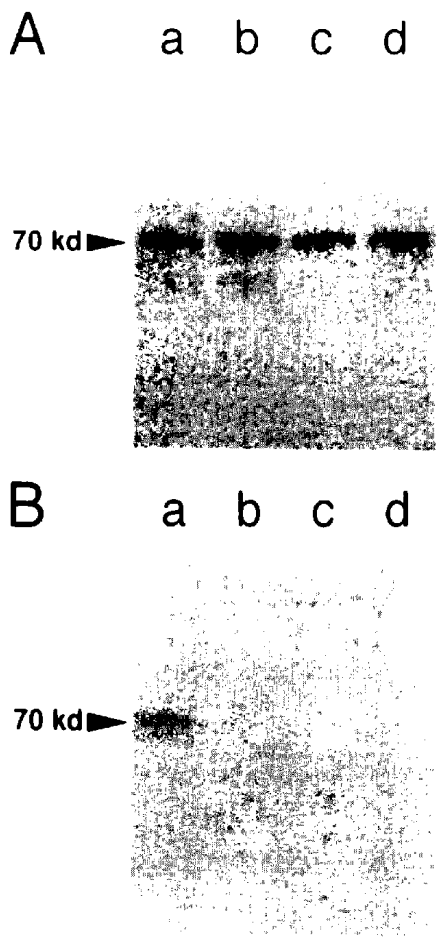


Fig. 3. Immunoblot analysis of COX isozymes in the sponge granuloma without growth factor stimulation. A, anti-COX-1; B, anti-COX-2. Lane a, day 4; Lane b, day 7; Lane c, day 9; Lane d, day 14. The position of the 70-kd molecule is indicated.

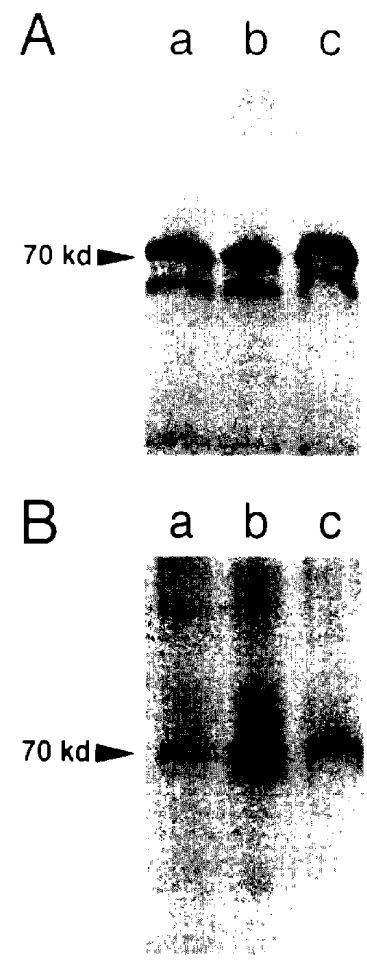


Fig. 4. Immunoblot analysis of COX isozymes in the sponge granuloma on day 4 with growth factor stimulation. A, anti-COX-1; B, anti-COX-2. Lane a, bFGF (100 ng/site/day); Lane b, bFGF (1000 ng/site/day); Lane c, EGF (1000 ng/site/day). The position of the 70-kd molecule is indicated.

infiltrated inflammatory cells.

The same positive staining was mainly observed in the endothelial cells and the fibroblast-like cells in the EGF-treated sponge granuloma.

Effects of COX inhibitors on angiogenesis in sponge granuloma

Figure 6 shows that the angiogenesis (day 4) enhanced with EGF (1000 ng/site) was significantly inhibited with indomethacin, returning to the level observed in rats without growth factor treatment. The selective COX-2 inhibitor NS-398 also inhibited the EFG-stimulated (1000 ng/site) angiogenesis to the same degree as indomethacin.

The angiogenesis enhanced with bFGF (100 ng/site) was also inhibited either by indomethacin or by NS-398 and retreated to the level seen in rats without bFGF. The angiogenesis induced by a higher dose of bFGF (1000 ng/site) was inhibited by NS-398 at two different doses. Indomethacin also inhibited the angiogenesis with the same potency as NS-398.

There was no marked and significant reduction in the weights of the granuloma tissues after the treatment with NS-398, although indomethacin significantly reduced the weights of these tissues (Table 2).

The angiogenesis in sponge implants stimulated by 4 days injection of bFGF (1000 ng/site) was significantly inhibited by a 2-day administration of NS-398 (Fig. 7). The inhibitory effect on angiogenesis of NS-398 administered for two days (day 1 and day 2) was more evident than that for the later two days (day 3 and day 4).

There was no significant reduction in the weights of the granuloma tissues after either 2-day treatment with NS-398 (Table 3).

Angiogenesis, which gradually developed over 14 days without growth factor treatment, was significantly sup-

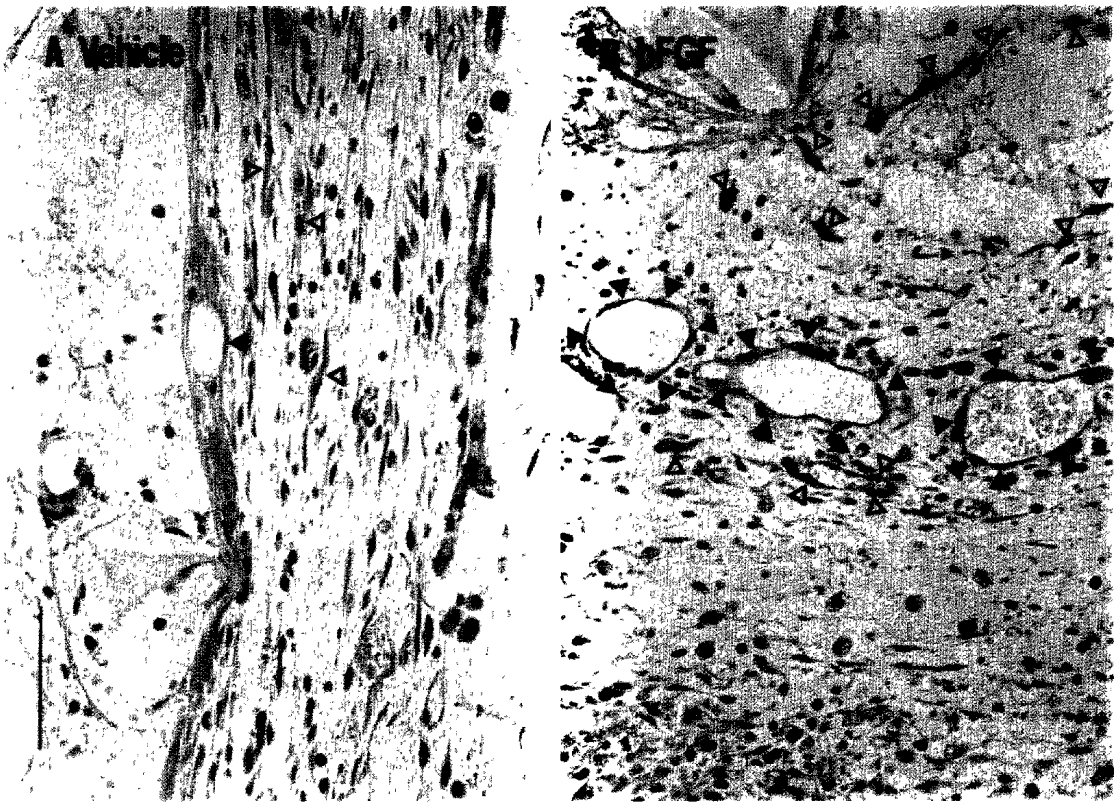
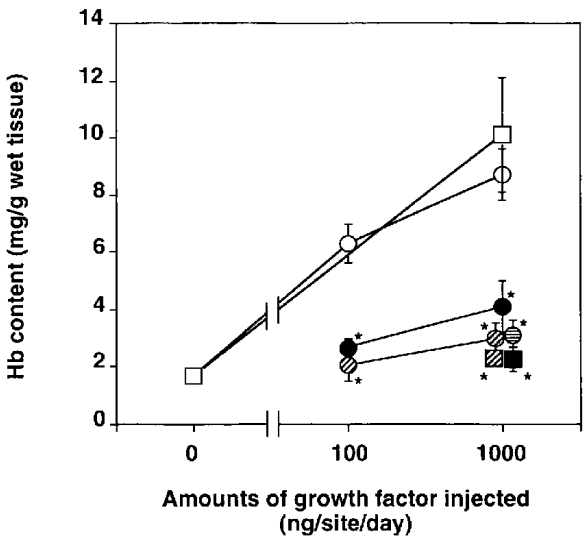


Fig. 5. Immunohistochemically stained sections of the sponge granuloma on day 4 with and without bFGF injections (1000 ng/site/day). Panel A shows results from immunohistochemical studies using anti-COX-2 antibody without bFGF. Panel B shows results of stimulation with bFGF. The bar represents 50 μm for both panels.

pressed by the prolonged (for 14 days) administration of NS-398 (Fig. 8).

There was no significant reduction in the weights of the granuloma tissues after 14 days of treatment with NS-398 (Table 4).



DISCUSSION

To our knowledge, this is the first paper in which the expression of an inducible COX isoform, COX-2, was detected in the granuloma tissues during neovascularization in the sponge implant model. COX is a key enzyme of prostaglandin formation, and two isozymes, COX-1 and COX-2, have been identified (11). COX-2 was reported to be induced in the cultured cells in response to various stimuli, including administration of lipopolysaccharide, cytokines (18), promotor phorbol ester (19) and growth factor (20). Even in in vivo models, the induction

Fig. 6. Effects of COX inhibitors on hemoglobin concentration in sponge granuloma at day-4 stimulated with growth factors. NS398 (NS) or indomethacin (IND) was administered orally for 4 days. Basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) was injected into the sponge implants once a day for 4 days. ○, bFGF+Vehicle; ●, bFGF+NS (3 mg/kg/8 hr); ◍, bFGF+NS (30 mg/kg/8 hr); ⊘, bFGF+IND (3 mg/kg/8 hr); □, EGF+Vehicle; ■, EGF+NS (3 mg/kg/8 hr); ▨, EGF+IND (3 mg/kg/8 hr). Each value represents the mean±S.E.M. from 6 to 10 animals. *$P<0.05$ vs vehicle control (open circles and open squares).

Table 2. Effects of NS398 or indomethacin on weights (mg) of sponge granuloma stimulated with EGF or bFGF

Treatment	Drugs		
	Vehicle	NS	IND
EGF (1000)	554±66	525±50	365±37*
bFGF (1000)	943±131	849±139	—

Each value represents the mean±S.E.M. from 6–8 animals. EGF (1000), EGF (1000 ng/site/day); bFGF (1000), bFGF (1000 ng/site/day); NS, NS398; IND, indomethacin. *P<0.05, comparison with the value from vehicle-treated rats on day 4.

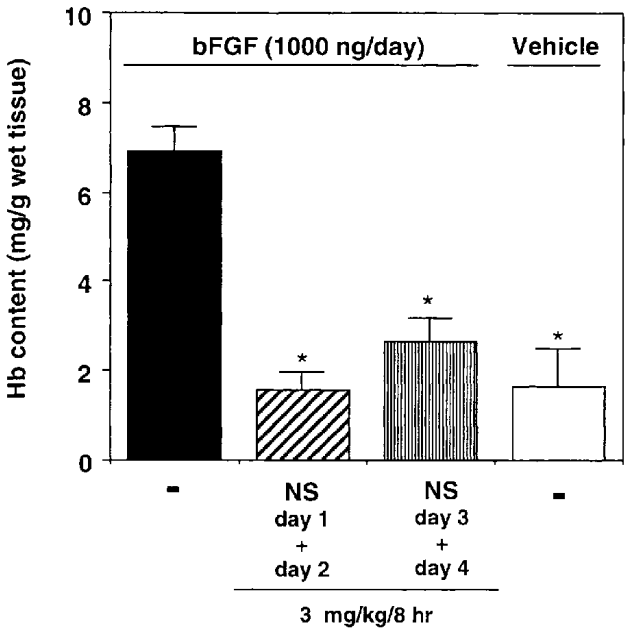


Fig. 7. Effects of 2-day administration of NS398 on hemoglobin contents of sponge implants at day 4 on stimulation with bFGF. Basic fibroblast growth factor (bFGF, 1000 ng/site/day) was injected into the sponge implants for 4 days. NS398 (NS) was administered orally for 2 days. Each value represents the mean± S.E.M. from 6 to 10 animals. *P<0.05 vs bFGF-treated control (closed column).

Table 3. Effects of 2-day administration of NS398 on weights (mg) of sponge granuloma stimulated with bFGF

Treatment	Drugs		
	Vehicle	NS (day 1 & day 2)	NS (day 3 & day 4)
bFGF (1000)	690±61*	518±82	518±48
No treatment	477±40	—	—

Each value represents the mean±S.E.M. from 4 animals. bFGF (1000), bFGF (1000 ng/site/day); NS, NS398 (3 mg/kg/8 hr) for 2 days. *P<0.05, comparison with the value from rats under no treatment on day 4.

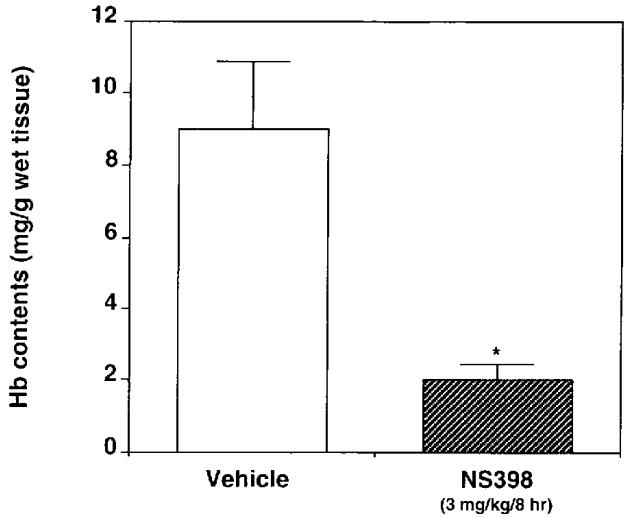


Fig. 8. Effects of 14-day administration of NS398 on hemoglobin contents of sponge implants at day 14 without growth factor. Substances orally administered for 14 days: NS398 or vehicle solution (Vehicle). Each value represents the mean±S.E.M. from 8 animals. *P<0.05 vs vehicle control (open column).

Table 4. Effects of NS398 on weights (mg) or sponge granuloma (day 14) without growth factor treatment

	Drugs	
	Vehicle	NS
No treatment	1155±156	784±139

Each value represents the mean±S.E.M. from 6 animals. NS, NS398.

of COX-2 was also reported, and the roles of COX-2 in prostaglandin formation in pathophysiological states such as pleurisy (21, 22), air pouch granuloma (23, 24), food pad edema (25), arthritis (26, 27), the repair stage of gastric ulcer (28), and proliferation of the uterine endometrium (12) have been discussed. However, the roles of COX-2 in angiogenesis have not been fully investigated and still require clarification.

The assays for angiogenesis that are presently available make use of the hamster cheek pouch (29), rabbit corneal implants (30) or chick chorioallantoic membrane (31). However, none of these procedures or their modifications is ideal, as was pointed out by Vallee and Riordan (32), because it is often difficult to quantify or observe vascular growth continuously. Anrade et al. reported a method for quantitative in vivo studies of angiogenesis using subcutaneous implantations of sterile polyester sponges in rats (33). They determined the amounts of neovascularization in terms of the blood flow in the implants as the sponges become vascularized. The blood flow in the im-

plants was measured in terms of percent ^{133}Xe clearance after this radioisotope was injected into the sponge (33). Alternatively, angiogenesis in the granuloma tissues can be estimated by the amount of dye injected intravenously (34). The hemoglobin content of the sponge granuloma tissues is also a good marker for angiogenesis in this model (14). With the quantitative approach in which the hemoglobin concentrations in the granuloma tissues were measured, we estimated the time course of the angiogenesis after implantation, and detected enhanced neovascularization of the sponge implants in animals administered growth factor (Fig. 2). Furthermore, dose-dependency in the enhancement of angiogenesis was observed with bFGF treatment (Fig. 2).

In the present study, we first succeeded in detecting COX-2 in the sponge granuloma tissues by Western blot analysis 4 days after implantation (Fig. 3B). In the experimental model of mouse gastric ulcer, the increase in COX-2 gene expression was detected 5 days after the induction of gastric ulcer lesions (28). Expression of COX-2 was also confirmed in the endometrium, in which the thickness of tissues was increased during estrus (12). It is plausible that during the repair of the ulcerative tissues and the proliferation of the endometrium, the neovascularization in these tissues is enhanced. We therefore studied the role of induced COX-2 in angiogenesis.

The level of COX-2 detected in sponge granuloma was apparently stimulated by EGF or bFGF (Fig. 4B). It has been frequently reported that growth factors, such as bFGF, EGF and transforming growth factor alpha (TGFα), increase the expressions of COX-2 in several types of cultured cells (35–38). Even in this in vivo model, the induction of COX-2 was enhanced with EGF and bFGF (Fig. 4B). The intense positive staining obtained in our immunohistochemical studies for immunoreactive COX-2 in the vasculatures of granuloma tissues under EGF or bFGF treatment also confirmed the increased induction of COX-2 (Fig. 5). This was also true in the case of the joints of rheumatoid arthritis patients (26). Although it was previously reported that the positive staining of COX was identified using a non-selective antibody for isozymes on the endothelium of vessels in the synovial tissues of the affected joints, a more recent report using a specific COX-2 antibody revealed that the positive stain in the patient synovial tissues was also located on the endothelium (27). These previous reports were consistent with the findings of our present study on the localization of the induced COX, COX-2.

After EGF and bFGF treatment, the thickness of the capsules of the sponge implants was increased in parallel with the increases in hemoglobin concentration (Figs. 1 and 2). The increased accumulation of collagen fibers in the granuloma tissues by the growth factor were identified by using van Gieson's staining (Fig. 1).

To evaluate the roles of COX-2 induced in the present angiogenesis model, we used a selective COX-2 inhibitor (17). It has already been reported that prostaglandin E_1 and prostaglandin E_2 enhanced the angiogenesis in vivo models (6, 7). Since the administration of prostaglandins caused the enhanced angiogenesis, the COX-2 detected in this model may augment neovascularization through prostaglandin formation. In our preliminary experiments, prostaglandin E_2 (370–510 pg/sponge) was detected in the sponge implants, even without stimulation of EGF or bFGF. A previous report that the angiogenesis in sponge implantation enhanced by EGF or bFGF was suppressed by a non-selective COX inhibitor naproxicen supported this possibility, since not only COX-1 but also COX-2 are inhibited by naproxicen (39). In the present experiment, the non-selective inhibitor indomethacin also markedly suppressed the stimulated angiogenesis, as determined from the hemoglobin content of the sponges (Fig. 6), and simultaneously caused a marked reduction in the weight of the granuloma tissues (Table 2). Furthermore, the stimulated angiogenesis was markedly reduced with the selective COX-2 inhibitor NS-398, which was equipotent to indomethacin (Fig. 6). Since a lower dose of NS-398, which had the same effect as a high dose, was equipotent to indomethacin (Fig. 6), it is plausible that COX-2, but not COX-1, has a role in neovascularization in this model under EGF or bFGF treatment. The weights of the granuloma tissues were not significantly reduced with NS-398 (Table 2), although angiogenesis was significantly reduced. This suggested that the increase in fibrous tissues after stimulation with bFGF or EGF may be independent of the neovascularization. The constitutive COX-1 may be involved in the enhanced encapsulation of this sponge granuloma, because of the significant effect of indomethacin on the weight of the granuloma tissues (Table 2). Since prostaglandin E_2 is reported to increase the expression of vascular endothelial growth factor (VEGF) (40, 41), insulin-like growth factor I (IGF-I) (42), and TGFα (43, 44), some growth factors may be involved in this sponge implantation model, especially during stimulation by bFGF and EGF.

In the glanuloma tissues, the infiltration of mononuclear cells accompanied by a few polymorphonuclear cells was observed. These cells may generate other metabolites of arachidonate such as leukotrienes. It was reported that leukotriene C_4, but not B_4 and D_4, stimulated tube formation of endothelial cells, which were cultured in the layers of collagen gels (45). The possible involvement of leukotrienes in the angiogenesis in the sponge glanuloma can not be ruled out in the present experiments.

The slow development of angiogenesis in the non-stimulated sponge implants was further significantly im-

peded with a prolonged administration of NS-398 (Fig. 8). Because of the toxicity of indomethacin, we did not administer it to rats for a long time. The low toxicity of NS-398 allowed us to administer it to rats and to evaluate its inhibitory effects on angiogenesis. We detected COX-2 in non-stimulated sponge implants only at day 4 by Western blot analysis (Fig. 3B), suggesting the possibility that the early stage of angiogenesis may be important and may be enhanced by the increased prostaglandin formation by COX-2. The fact that the early 2-day administration of a COX-2 inhibitor inhibited the angiogenesis markedly (Fig. 7) was consistent with this possibility. However, the anti-serum for COX-2 used in the present experiments was originally prepared for mice, not for rats. In the granuloma tissues at 10 and 20 days after implantation into the subcutaneous tissues of mice, we can detect COX-2 using this antibody, even without growth factor treatment (results will be published separately). Thus, it may be another possibility that we failed to detect the COX-2 throughout the experimental period. It is possible that COX-2 can be detected throughout the experimental period with a more sensitive assay procedure than that used in the present experiment.

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