Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice

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Sawaoika, Hitoshi, Sunao Kawano, Shingo Tsuji, Masahiko Tsuji, Edhi S. Gunawan, Yoshiyuki Takei, Kouichi Nagano, and Masatsugu Hori. Cyclooxygenase-2 inhibitors suppress the growth of gastric cancer xenografts via induction of apoptosis in nude mice. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G1061–G1067, 1998.—To clarify the role of mitogen-inducible cyclooxygenase (COX-2) in the development of malignant tumors, we investigated the effects of COX-2 inhibitors on the growth of gastric cancer xenografts in nude mice in vivo. MKN45 gastric cancer cells (5 × 10⁶ cells/animal) that overexpress COX-2 were inoculated subcutaneously into athymic mice. NS-398, a specific COX-2 inhibitor, or indomethacin, a nonspecific COX-2 inhibitor, was administered orally to animals every day for 20 days. These drugs reduced the tumor volume significantly. Immunohistochemistry using bromodeoxyuridine, nick end labeling, and electron microscopy showed that NS-398 induced apoptosis in cancer cells in a dose-dependent manner and inhibited cancer cell replication slightly. Indomethacin also induced apoptosis and suppressed replication of tumor cells. There was a significant negative correlation between tumor volume and apoptotic cell number within the tumor. These results are consistent with the hypothesis that COX-2 inhibitors suppress growth of gastric cancer xenografts mainly by inducing apoptosis and suppressing replication of the neoplastic cells. It follows that COX-2 plays an important role in the development of gastric cancer.

GASTRIC CANCER is a major cause of death throughout the world, even though its prognosis has improved yearly due to advances in diagnostic and surgical techniques. According to a report by the World Health Organization (35), in 1993 gastric cancer was the fourth leading cause of cancer death in the United States, killing more than 8,000 people that year. In Japan, gastric cancer was found to be the second leading cause of cancer death in 1994, with over 30,000 people killed by gastric cancer that year (29). In a 1993 mortality study reported that regular aspirin use may reduce the risk of fatal colon cancer (31). It is unclear whether this is due to the inhibition of the growth of cyclooxygenase (COXs; also referred to as prostaglandin endoperoxide synthases, EC 1.14.99.1) catalyze conversion of arachidonic acid to prostaglandin G₂/H₂ and consist of at least two isozymes, i.e., constitutive COX-1 and mitogen-inducible COX-2 (3, 5). Human gastric carcinoma tissue expresses significantly higher levels of cox-2 mRNA than noncancerous adjacent tissue (23). COX-2 is overexpressed in neoplastic tissues but not in normal tissues adjacent to the neoplasm in most patients with colon cancers (6). We have reported that COX-2 is expressed in several cell lines derived from human gastrointestinal adenocarcinomas (32). On the other hand, in vitro studies indicate that overexpression of COX-2 results in differentiation, adhesion to extracellular matrices, and inhibition of programmed cell death of rat intestinal cells (33). However, the role of COX-2 in the growth of cancer cells in vivo has not been fully clarified.

To clarify the role of COX-2 in the growth of neoplastic tissue, we investigated the effects of NS-398 and indomethacin on the growth of gastric cancer xenografts transplanted into athymic mice. NS-398 [N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide is a sulfonamide derivative that inhibits COX-2 specifically with an IC₅₀ of ∼30 nM. It does not affect COX-1 activity at concentrations exceeding 100 µM (7, 8, 10, 15). Per os administration of NS-398 lowers prostanoid production in inflammatory exudate at a 50% inhibitory dose (ID₅₀) of ∼0.2 mg/kg; however, in noninflammatory tissues in vivo, it inhibits prostanoid production only minimally even at doses >200 mg/kg (7). Indomethacin inhibits both COX-1 (IC₅₀ = 100 nM) and COX-2 (IC₅₀ = 900 nM) but only minimally at concentrations exceeding 100 µM (7). Indomethacin inhibits both COX-1 (IC₅₀ = 100 nM) and COX-2 (IC₅₀ = 900 nM) in vitro (10) and abolishes prostanoid production of both inflamed and noninflamed tissues at ID₅₀ values ∼0.2 mg/kg in vivo (7). We used MKN45 cells established from a patient with poorly differentiated adenocarcinoma of the stomach (17), because we found that this cell line in vitro overexpresses human COX-2 with expression of human COX-1 (32). We also investigated the effects of these inhibitors on cell replication, necrosis, and apoptosis in gastric cancer xenografts in athymic mice.

MATERIALS AND METHODS

Agents

NS-398 was a generous gift from Dr. S. Higuchi (Taisho Pharmaceutical, Tokyo, J apan). Indomethacin and gum arabic were purchased from Sigma Chemical (St. Louis, MO) and Nakalai Tesque (Kyoto, J apan), respectively. NS-398 and indomethacin were suspended with gum arabic (50 mg/kg) in water, using an agate mortar. Other reagents were of analytic purity.
grade and obtained from Wako (Osaka, Japan), unless otherwise described.

Animals

Animal experiments were performed according to the guidelines of the Committee on Experimental Animals of Osaka University. We purchased 78 female athymic mice (BALB/c nu/nu; 5 wk old; 12–16 g) from Nippon Clea (Tokyo, Japan). The animals were maintained under specific pathogen-free conditions using a laminar air-flow rack and had continuous free access to sterilized food (gamma ray-irradiated food, CL-2; Nippon Clea) and autoclaved water. Experiments were started after 1 wk of acclimatization.

Tumor Growth

The MKN45 human gastric cancer cell line (17) was supplied by the Japanese Cancer Research Bank (Tokyo, Japan). The cells were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37°C in RPMI 1640 medium (LifeTech, Grand Island, NY) supplemented with 10% heat-inactivated FCS (BioWhittaker, Walkersville, MD). The medium was changed every 3 days.

Subconfluent MKN45 cells were dissociated with 0.25% trypsin and 1 mM EDTA (LifeTech) and suspended in PBS at a density of 5 × 10^7 cells/ml. Each mouse was inoculated with MKN45 (5 × 10^6 cells/animal) subcutaneously on the left side of the back on day 0. The animals were randomly divided into six groups (groups A–F; 8 animals/group) immediately after inoculation and received one of the following treatments. Oral administration of gum arabic (50 mg/kg), NS-398 (10, 30, or 100 mg/kg body wt) or a nonulcerogenic dose (3 mg/kg body wt) of indomethacin (8) was initiated in groups A–E, respectively, 24 h after tumor inoculation (day 1) and repeated daily for 3 wk (until day 20). In group F, daily administration of gum arabic was begun at day 1 and then replaced by daily administration of NS-398 (100 mg/kg) 10 days after tumor inoculation (until day 20). The shortest and longest diameter of the tumor were measured with calipers at 5-day intervals, and tumor volume (mm^3) was calculated using the following standard formula (12): (the shortest diameter)^2 × (the longest diameter) × 0.5. There were no differences in body weight among the groups throughout the experiment.

Histopathological and Immunohistochemical Examinations

On day 20, mice were given bromodeoxyuridine (BrdU; Sigma Chemical; 50 mg/kg body wt) by intraperitoneal injection. Three hours later, they were killed under ether anesthesia. All tumors were dissected from the body and divided along the longest diameter with a surgical knife. Half of each specimen was embedded in cryocompound (TISSUE-TEK; Miles, Elkhart, IN) and immediately immersed in liquid nitrogen. The frozen specimens were sectioned with a cryotome at a thickness of 6 µm. The other half of each specimen was fixed in 10% phosphate-buffered Formalin, embedded in paraffin, and sectioned at a thickness of 4 µm. These sections were partly dissected, prefixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and used for electron microscopic analysis as described below.

Tumor histology. Tissue sections were deparaffinized and stained with hematoxylin and eosin. The slides were coded and examined by a pathologist without knowledge of the group to which the specimen belonged. On the slide, four radii were set on the tumor section at right angles, and then each radius was divided by 4. The histology of each tumor was photographed at five regions of interest (0.175 mm²); the center of the specimen, the farthest extent of the first radius, the outer quarter of the second radius, the center of the third radius, and the inner quarter of the fourth radius (Fig. 1). The degree of necrosis of the tumor was scored according to the method of Takei et al. (30). In brief, necrotic tissue observed in

Fig. 1. Diagram of region of interest. 1, The center of the specimen; 2, the farthest extent of the first radius; 3, the outer quarter of the second radius; 4, the center of the third radius; 5, the inner quarter of the fourth radius.

Fig. 2. A: effect of NS-398 and indomethacin on growth of human gastric cancer xenografts in athymic mice. MKN45 human gastric cancer cells (5 × 10^6 cells/animal) in PBS were inoculated subcutaneously into the left side of the back of each nude mouse on day 0. NS-398 (10, 30, and 100 mg/kg body wt) and indomethacin (3 mg/kg body wt) were suspended in water with gum arabic and given orally 24 h after tumor inoculation (day 1) for 3 wk (until day 20). In the control group, gum arabic suspended in water alone was administered in the same manner. B: effect of NS-398 on the growth of MKN45 xenografts in athymic mice. In this experiment, oral administration of 100 mg/kg of NS-398 was started 10 days after tumor inoculation and continued for 10 days. Tumor volume (mm^3) was measured with calipers and calculated using the following standard formula: (the shortest diameter)^2 × (the longest diameter) × 0.5. *P < 0.05 vs. control; **P < 0.01 vs. control by one-way ANOVA followed by Scheffe’s F procedure.
1–20% of the regions of interest was scored as 1. Necrosis found in 21–40%, 41–60%, 61–80%, and 81–100% of the regions of interest was scored as 2, 3, 4, and 5, respectively. If necrosis occupied <1% of the regions of interest, the score was 0. Then the mean of the regions of interest was calculated and designated as the score of the tumor.

COX-2 expression. Cryosections were fixed in cold acetone, incubated with a primary antibody against COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and treated with a secondary antibody. They were stained according to the avidin-biotin-peroxide complex (ABC) method using a commercial kit (Vecstatin kit; Vector Laboratories, Burlingame, CA) and visualized by 3,3′-diaminobenzidine (DAB) (Vecstatin DAB kit, Vector Laboratories). Subsequently, the specimens were counterstained with hematoxylin. Another section was incubated with the COX-2 antibody preabsorbed with human COX-2 peptide (Santa Cruz), treated with the secondary antibody, and stained by the ABC-DAB method.

Cell replication. Specimens were deparaffinized and incubated with a primary antibody against BrdU (Becton-Dickinson Immunocytometry Systems, San Jose, CA). The specimens were then treated with the secondary antibody, stained by the ABC method using a Vecstatin kit, and microscopically examined at five observation points. The number of BrdU-positive cells was counted at the five regions of interest as described above. The BrdU labeling index was calculated as follows: labeling index (%) = (number of cells labeled with BrdU/total cell number) × 100.

Apoptosis. Terminal deoxynucleotidyl transferase-mediated dUDP nick end labeling (TUNEL) (9) was performed using a commercial kit (Apoptag Plus; Oncor, Gaithersburg, MD) (11). The number of apoptotic cells positive for TUNEL staining was counted at the five regions of interest. The apoptotic index was calculated as follows: apoptotic index (%) = (apoptotic cell number/total cell number) × 100.

Histology of tissues other than the cancer xenograft. The lungs, esophagus, stomach, jejunum, colon, liver, and kidneys were also dissected, fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned at a thickness of 4 μm. For histological examination, tumors were stained with hematoxylin and eosin. For detection of apoptotic cells, TUNEL was performed.

Prostaglandin Levels in Tumors

To confirm that COX-2 inhibitors influence prostaglandin synthesis in the tumor, the other 30 mice were inoculated with 5 × 10⁶ MKN45 cells/animal. The animals were randomly divided into five groups (groups A–E; 6 animals/group). The received gum arabic (50 mg/kg), NS-398 (10, 30, or 100 mg/kg body wt), or indomethacin (3 mg/kg) from days 1 to 20, respectively. Tumors were dissected on day 20, divided immediately into ~1-mm³ pieces in saline containing 10⁻⁴ M indomethacin and 10 mM disodium salt of EDTA, and homogenized in 4 ml of cold ethanol with Polytron-Aggregate (Kinematica, Lucerne, Switzerland). The homogenized tissue in ethanol was centrifuged at 1,630 g for 15 min. Prostaglandin E₂ and 6-ketoprostaglandin F₁α were measured in the supernatant by RIA using commercial kits (prostaglandin E₂ and 6-ketoprostaglandin F₁α; NEN Life Science, Boston, MA). The pellet was dissolved in 5 ml of 1 M NaOH and used for protein assay by the method of Lowry et al. (14). Prostaglandins were expressed as picograms per milligram of protein.
Statistical Analysis

Data are expressed as means ± SE and were compared by one-way ANOVA followed by Scheffe's F procedure or by nonparametric ANOVA. The relationships between the tumor volume and the apoptotic index and between the tumor volume and the BrdU-labeling index were examined by simple linear regression. The relationship between the tumor volume and the necrotic index was examined by Spearman's regression test. The criterion for statistical significance was taken as $P < 0.05$.

RESULTS

Effect of NS-398 and Indomethacin on Tumor Growth

Figure 2A shows the effects of NS-398 and indomethacin on the growth of human gastric cancer xenografts in athymic mice. Oral administration of NS-398 (10, 30, and 100 mg/kg) and indomethacin (30 mg/kg) inhibited BrdU incorporation significantly compared with the control group. Indomethacin and NS-398 (30 and 100 mg/kg) inhibited BrdU incorporation significantly compared with the control group. *$P < 0.01$ vs. control by one-way ANOVA followed by Scheffe's F procedure.

Histological and Immunohistochemical Findings

Tumor histology. Histopathological examination (by hematoxylin and eosin staining) revealed no apparent differences in morphology of the tumor cells among all groups studied. Tumor cells from all xenografts had clear cytoplasm and pale, round nuclei and did not contain mucus (Fig. 3A). There were no significant differences in the histological score of necrosis among the groups (Fig. 4A).

COX-2 expression. Immunohistochemical examination revealed COX-2-immunoreactive material in the xenografts of MKN45 cells in all groups studied. COX-2 immunoreactivity was particularly strong at the nuclei of neoplastic cells (Fig. 3B). Neutralization with pure COX-2 peptide confirmed the specificity of the antibody against COX-2 (Fig. 3C). Treatment with NS-398 or...
Indomethacin did not influence the expression of COX-2 immunoreactivity in MKN45 xenografts.

Cell replication. BrdU labeling indexes of the tumors were investigated in each group. NS-398 (30 and 100 mg/kg) and indomethacin significantly inhibited the BrdU labeling index by 20.5%, 24.2%, and 21.7%, respectively (Fig. 4B). NS-398 suppressed the BrdU labeling index of the tumor in a dose-dependent manner.

Apoptosis. Apoptotic indexes of gastric cancer xenografts were investigated in each group. NS-398 (10, 30, and 100 mg/kg) and indomethacin (3 mg/kg) significantly induced apoptosis of MKN45 cells within the xenograft by 2.6-, 3.3-, 5.1-, and 4.3-fold, respectively (Fig. 4C). Transmission electron microscopy also demonstrated condensed nuclei, apoptotic bodies, and shrunken cancer cells, morphological indications of apoptosis, in the animals treated with either NS-398 or indomethacin (Fig. 5).

Histology of tissues other than the cancer xenograft. Neither NS-398 (10, 30, or 100 mg/kg) nor indomethacin (3 mg/kg) caused damage in the lungs, esophagus, stomach, jejunum, colon, liver, or kidneys of the host animals. TUNEL-positive apoptotic cells were rarely found in these organs or were not increased compared with control animals (data not shown).

Relationship between tumor volume and tumor cell necrosis, apoptosis, and cell replication. There was a negative correlation between tumor volume and apoptotic cell number (Fig. 6A; \( r = 0.698, P < 0.01 \)) and a positive correlation between tumor volume and the BrdU-labelling index (Fig. 6B; \( r = 0.762 \)). It should be noted that the growth of the tumors was suppressed even when their BrdU labeling indexes were >23%. There was no relationship between tumor volume and the histological score of necrosis (data not shown).

Prostaglandin Levels in Tumors

Prostaglandin E2 and 6-ketoprostaglandin F1α levels of the tumors were significantly decreased in groups treated with NS-398 and indomethacin compared with the gum arabic-treated control group. Tissue levels of these prostaglandins in the xenografts were decreased in a dose-dependent manner in groups treated with NS-398 (10, 30, and 100 mg/kg) (Fig. 7).

**DISCUSSION**

The present study shows that MKN45 cells transplanted into athymic mice express COX-2 and form solid tumors on the backs of the animals. The results also demonstrated that NS-398, a specific inhibitor of COX-2, repressed growth of the gastric cancer xenograft in a dose-dependent manner. Indomethacin, an inhibitor for both COX-1 and COX-2 (10), also suppressed tumor growth. Both NS-398 and indomethacin suppressed prostaglandin production by the xenograft significantly, indicating that orally administered COX inhibitors actually inhibit the COX-2 enzyme of the xenografts. The present study therefore demonstrates that oral administration of specific and nonspecific inhibitors for COX-2 significantly suppresses the growth of xenografts of MKN45 in athymic mice. The data indicate that COX-2 expression plays an important role in the growth of gastric cancer xenografts in vivo.

There are several mechanisms that participate in the growth of malignant tumors. Proliferation of tumor cells causes expansion of tumor volume. On the other hand, data show that tumor volume is inversely correlated with apoptotic cell number and positively correlated with BrdU-positive cells (Fig. 6). This suggests that apoptosis is a key factor in the growth of gastric cancer xenografts in vivo. Additionally, the levels of prostaglandins in the tumors were decreased in a dose-dependent manner in groups treated with NS-398 (10, 30, and 100 mg/kg), indicating that COX-2 inhibitors may be effective in suppressing tumor growth.
hand, death of tumor cells by necrosis and apoptosis reduces the size of the tumor. In the present study, we investigated the effects of NS-398 and indomethacin on these phenomena in xenografts of MKN45 in athymic mice. The results showed that neither NS-398 nor indomethacin caused increased necrosis within the tumor. Moreover, indomethacin and NS-398 slightly but significantly lowered the BrdU labeling index in the tumor cells of xenografts in nude mice. One may expect that decreases in cell replication by these agents play a primary role in suppression of tumor growth. Indeed, the volume of the xenografts correlated positively with BrdU labeling indexes. These results are compatible with previous findings that COX-2 inhibitors suppress proliferation of MKN45 cells in vitro (32). However, growth of the tumors might be defined not only by cell proliferation but also by regression of cells by cell death. On the other hand, NS-398 increased the number of TUNEL-positive apoptotic cells in the tumor in a dose-dependent manner. Indomethacin also increased the number of apoptotic cells in the gastric cancer xenograft. Furthermore, electron microscopy showed condensation and fragmentation of the nuclei of cancer cells in mice treated with NS-398 or indomethacin, confirming the occurrence of apoptosis in tumor cells. Neither NS-398 nor indomethacin influenced the degree of necrosis of the tumors. In a certain tissue, occurrence of DNA fragmentation, a process of apoptosis, is followed by apparent death of the cells, which are removed from the tissue within several hours (1, 2). Bursch et al. (1, 2) reported that only 2% or 3% of the apoptotic cells in the tissue resulted in regression of the tissue by 25%/day. Furthermore, the apoptotic index of the tumors correlated negatively with tumor volume (Fig. 6A), indicating that induction of apoptosis plays an important role in suppression of the growth of the tumor. Therefore, our results may suggest that expression of COX-2 plays an important role in tumor growth by inhibiting apoptosis and maintaining proliferation of gastric cancer cells.

Recently, Tsujii and DuBois (33) reported that overexpression of COX-2 prevented differentiation, cellular detachment from the extracellular matrix, and programmed cell death of nontransformed intestinal epithelial cells. They also showed that sulindac sulfide, another nonspecific inhibitor for COX-1 and COX-2, caused differentiation, cell detachment, and apoptosis in cell lines overexpressing COX-2 (33). Using the same cell lines, DuBois et al. (4) also showed that overexpression of COX-2 resulted in a delay in the G_{1} phase of cell replication. In contrast, a recent study by Shiff et al. (27) showed induction of apoptosis in HT-29 cells by more than 400 µM indomethacin, 1,000 µM naproxen, or 900 µM piroxicam in vitro. Furthermore, Shiff et al. (27) reported that other groups of nonsteroidal anti-inflammatory drugs (NSAIDs) failed to induce apoptosis in the same cells. Consequently, it is unclear how the COX inhibitors affect cell kinetics of malignant tumors both in vitro and in vivo. Although Sheng et al. (26) recently demonstrated that a selective inhibitor of COX-2 suppressed growth of HCA-7 colon cancer cells in vivo and in vitro, the precise mechanisms for these phenomena were not clarified. In contrast, our study offers evidence for the first time that selective and nonselective inhibitors of COX-2 at reasonable doses suppress the growth of tumors transplanted in athymic mice by inducing apoptosis and suppressing cell replication in vivo.

Because MKN45 is a cell line established from a patient with poorly differentiated gastric adenocarcinoma, one may suspect that the present results may not be attributable to gastric adenocarcinoma in general. However, poorly differentiated adenocarcinoma, one of the most common types of gastric cancer, accounts for ~55% of gastric cancer cases (28, 34). In addition, gastric cancer cell lines derived from cancer with other types of histology, such as MKN28 (moderately differentiated tubular adenocarcinoma) and KATO III (signet ring cell carcinoma), also express cyclooxygenase-2 (cyclooxygenase-2) mRNA (32). Furthermore, human gastric carcinoma tissue expressed significantly higher levels of cyclooxygenase-2 mRNA than did samples without carcinoma (23). Therefore, the present results may be applicable to gastric carcinoma in general.

COX inhibitors have been used as NSAIDs for many years, but it was discovered only recently that they could be used to prevent the development of colon cancers. Inhibitors of this enzyme have been reported to lower the incidence of colon tumors induced by chemical carcinogens in rodent models (16, 18, 21, 22). Moreover, incidence of colon cancer has been reported to be lower in users of COX inhibitors, such as aspirin, than in control subjects not using the agents (24, 31). Sulindac, an NSAID, also inhibited the growth of intestinal polyps in patients with familial adenomatous polyposis (19). These reports have implicated potential usefulness of nonspecific inhibitors of COX in chemoprevention of colon cancers. However, nonspecific inhibitors of COX often cause adverse effects, such as gastrointestinal hemorrhage and ulceration. Currently, COX-2-specific inhibitors are expected to be nonulcerogenic and less toxic to the subjects. For example, Futaki et al. (8) reported that NS-398 did not lead to gastric damage at doses that cause analgesic and antipyretic effects in rats. Our results also showed that NS-398 did not cause any macroscopic or histological changes in tissues other than the gastric cancer xenograft at any dose used. However, at higher doses indomethacin is known to cause ulceration and necrosis in the stomach and jejunum (20, 25). The present study demonstrated that NS-398, a COX-2-specific inhibitor, suppressed the growth of gastric cancer xenografts as well as indomethacin, a nonspecific inhibitor of COX-2. Furthermore, our study showed that these inhibitors of COX could suppress the growth of tumors in which cells had already transformed to a poorly differentiated adenocarcinoma, in contrast to previous reports regarding their chemopreventive effects (16, 18, 19, 21, 22, 24, 31). The potential usefulness of COX inhibitors, and particularly that of COX-2-specific inhibitors, in chemotherapy for gastric cancers remains to be investigated in clinical studies.

In conclusion, we demonstrated that specific and nonspecific inhibitors of COX-2 suppressed growth of
tumor volume and cell replication and induced apoptosis of the human gastric cancer xenografts in athymic mice in vivo. Tumor volume was strongly associated with the degree of apoptosis of tumor cells, indicating that these agents inhibited tumor growth mainly by inducing apoptosis. Thus expression of this isoform of cyclooxygenase plays an important role in the development of gastric adenocarcinoma.

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