Meloxicam inhibits osteosarcoma growth, invasiveness and metastasis by COX-2-dependent and independent routes

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Cyclooxygenase-2 (COX-2) inhibitors exert antitumor activity via COX-2-dependent and independent pathways. We wished to evaluate the antitumor activity of meloxicam, a preferential COX-2 inhibitor, in osteosarcoma, the most common primary malignant bone tumor, and determine whether its antitumor effect is COX-2-dependent. COX-2 expression in the osteosarcoma cell lines MG-63, HOS and U2-OS was determined by real-time RT–PCR and western blotting. Subsequently, the inhibitory effects of meloxicam on osteosarcoma cell growth and invasiveness were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and matrigel invasion assays, respectively. Apoptotic activity was evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining and semi-quantification of Bax and Bcl-2 expression by real time RT–PCR and western blotting. Prostaglandin-E2 (PGE2) production in the presence and absence of meloxicam was analyzed by enzyme immunoassay, and to determine whether the effects of meloxicam are COX-2-dependent or independent, PGE2 was added to see if it reversed the effects of meloxicam. In addition, the effects of meloxicam on tumor growth and metastasis were evaluated in an in vivo mouse model using grafted LM-8 mouse osteosarcoma cells, together with immunohistochemical analysis for vascular endothelial growth factor in lung metastatic lesion. Meloxicam inhibited PGE2 production, proliferation and invasiveness especially in MG-63 cells, which express relatively high levels of COX-2. Only high concentrations of meloxicam caused apoptosis and upregulated Bax mRNA and protein in MG-63 cell culture. In contrast, meloxicam did not induce apoptosis in HOS and U2-OS cells, expressing relatively low levels of COX-2. Exogenous PGE2 reduced the effects of meloxicam on cell viability and invasiveness, but not its effect on Bax mRNA. In vivo, high doses of meloxicam suppressed LM-8 tumor growth and lung metastasis. Meloxicam, may have both COX-2-dependent and independent inhibitory actions on osteosarcoma. Its effects are more prominent in osteosarcoma cells that have relatively high levels of COX-2.

Introduction

There are two isoforms of cyclooxygenase (COX), designated COX-1 and COX-2. COX-2 is expressed constitutively in many tissues, and seems to perform physiological functions (1). COX-2 is an inducible enzyme, associated with inflammatory disease and cancer (2). Many lines of evidence suggest that COX-2 plays a crucial role in various aspects of carcinogenesis. It is overexpressed in many types of malignant tumor, such as colorectal (3), prostate (4), breast (5) and lung cancer (6), and pharmacological studies suggest that it is a useful therapeutic target. Selective inhibitors of COX-2 reduce the formation, growth and metastasis of experimental tumors (7,8), and genetic studies support a cause–effect connection between COX-2 and tumorigenesis. Transgenic mice overexpressing human COX-2 in their mammary glands developed focal mammary gland hyperplasia, dysplasia and metastatic tumors (9), and epidemiological studies have revealed a decreased risk of colon cancer in people who regularly take COX-2 inhibitors (10,11).

Osteosarcoma is the most common primary malignant bone tumor (12); there are three main types, osteoblastic, chondroblastic and fibroblastic, depending on the element affected. It is also one of the most heterogeneous of human tumors: its microscopic features vary between different lesions and even across areas of the same tumor. A better understanding of the basic biology of this tumor, such as its response to COX-2 inhibitors, may suggest novel modes of treatment. The prognosis for osteosarcoma has improved with the introduction of chemotherapy, but current response rates cannot be raised by increased dosage as drug resistance might limit further effectiveness. Therefore, there is a clear and critical need to develop new and alternative strategies for treating patients with osteosarcoma.

Meloxicam was developed as a new type of non-steroidal anti-inflammatory drug (NSAID) in 1977 at Boehringer Ingelheim, Germany, and has been commercialized clinically in more than 100 nations of the world. Its IC50 for COX-2 is approximately equal to its IC25 for COX-1. Hence it is categorized as a ‘preferential’ COX-2 inhibitor. The inhibitory actions of meloxicam on colorectal cancer cells (13) and non-small cell lung cancer cells (14) have been reported previously. Although there is considerable data on the use of this COX-2 inhibitor against cancer of the colon and the breast, there have been few studies of its use against osteosarcoma. Since osteosarcomas represent a group of heterogeneous tumors, and are reported to express COX-2 constitutively (15,16), it seemed worthwhile to examine the level of expression of COX-2 in osteosarcoma tissues, and to analyze the action of COX-2 inhibitors on osteosarcoma.
In this study, we determined the levels of expression of COX-2 in several osteosarcoma cell lines. We also examined the effect of meloxicam on cell growth, invasiveness and apoptosis, as well as on Bax and Bcl-2 expression, and showed that it inhibits growth of the cells and induces apoptosis. We found that COX-2-derived PGE2 stimulated the growth and invasiveness of osteosarcoma cells overexpressing COX-2 and that tumor formation by these cells was inhibited by meloxicam in a dose-dependent manner. Only high doses of meloxicam caused apoptosis, suggesting that the effect on apoptosis may involve a COX-2-independent pathway. In contrast, pro-apoptotic effect was not induced by meloxicam in other cell lines that expressed relatively low levels of COX-2. We also found that meloxicam inhibited tumor growth and reduced the number of lung metastasis formed by cells of the murine osteosarcoma cell line, LM-8, in an in vivo mouse model.

Materials and methods

Cell culture
Meloxicam was kindly provided by Boehringer Ingelheim, Germany. The human osteosarcoma cell lines, MG-63, HOS and U2-OS (purchased from the American Type Culture Collection, VA), and the murine osteosarcoma cell line, LM-8 (a kind gift from Dr A. Uchida), were grown as monolayers in Dulbecco’s modified Eagle’s medium (DMEM, Sigma Aldrich, MO) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate. The cultures were maintained in a humidified atmosphere with 5% CO2 at 37°C.

A 10 mM stock solution of meloxicam was diluted in DMEM (10% FBS) to the desired final concentration. All incubations were performed in the presence of 10% FBS.

PGE2 (Sigma Aldrich) was dissolved in 100% ethanol and added to the medium at a final concentration of 10 μM.

Real time RT–PCR analysis

Given that the anti-tumorigenicity effect of meloxicam is thought to depend on the levels of COX-2 expression in tumor cells, expression levels of COX-2 mRNA was determined in the human osteosarcoma cell lines MG-63, HOS, U2-OS and LM-8. Total cellular RNA was isolated using Trizol reagent (Life Technologies, NY) from subconfluent culture of MG-63, HOS, U2-OS or LM-8 cells, and used for reverse-transcription. 0.5 μg of total cellular RNA was converted to cDNA using Molony murine leukemia virus reverse transcriptase in the presence of 0.15 M dNTPs. All incubations were performed in the presence of 0.15 M dNTPs.

The mRNA levels were calculated permitting real-time monitoring of the PCR. The mRNA levels were calculated for each sample from the standard curve by the LightCycler Data Analysis software (Roche Molecular Biochemicals, Germany). The relative level of COX-2 mRNA in a sample was expressed as the percentage ratio of COX-2/GAPDH. The COX-2 and GAPDH primer pairs were as follows: Bax sense; 5'-GGCCCCAATACGTGTCATT-3' antisense; 5'-AGAGTGAGGAGGACATGGTGAC-3' (predicted PCR product of 465 bp).

To investigate the action of meloxicam on apoptosis, Bax and Bcl-2 mRNA levels were assayed also by semi-quantitative real-time RT-PCR. The Bax and Bcl-2 primer pairs were as follows: Bax sense; 5'-GCCCGCTAATCTGTTGCACT-3' antisense; 5'-GGTTGGTCTGGAGACATGGTGAC-3' (predicted PCR product of 236 bp).

Western blot analysis

Expression levels of COX-2 protein in MG-63, HOS and U2-OS cells were determined semi-quantitatively by western blot analysis. The effects of meloxicam on expression levels of Bax and Bcl-2 protein were also assessed by western blot. Cells were lysed, and protein was extracted with CellLyte™ M (Sigma Aldrich) containing a protease inhibitor cocktail (Sigma Aldrich). The concentration of the protein was calculated by BCA protein assay, and the protein (35 μg/lane) was fractionated by 10% SDS–PAGE and transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Hybond™ T Middle, Amersham Biosciences, UK). The membranes were incubated in 10% non-fat dried milk for 10 min and then probed with primary antibody for 1 h at 37°C. The primary antibodies used were goat anti-human COX-2 polyclonal antibody (Santa Cruz Biotechnology, CA) (1:100), rabbit anti-human Bcl-2 polyclonal antibody (Upstate, NY) (1:500) and mouse anti-human Bcl-2 monoclonal antibody ( Oncogene, CA) (1:50). After rinsing with phosphate buffered saline (PBS containing 0.1% Tween-20; polyoxyethylene sorbitan monolaurate; Sigma Aldrich), the membranes were incubated for 1 h at 37°C with horseradish peroxidase (HRP)-linked secondary anti-goat, anti-rabbit and anti-mouse IgG (Vector Laboratories, CA) (1:400) for COX-2, Bax and Bcl-2, respectively. The membranes were washed again, and the blots were visualized with enhanced chemiluminescence reagents (ECL™ western blotting detection reagents, American Biosciences, UK) and immediately photographed with a CCD digital scan camera (Cool Saver, Rise & ATTO Corporation, Japan). The luminescence densities of each band were calculated with CS Analyzer software (Rise & ATTO Corporation). The membrane was also blotted with anti-human β-actin mouse monoclonal antibody (Sigma, MO) (1:5000), and luminescence densities were calculated. Values were normalized to the values of β-actin of each sample.

Assay of cell viability

Invasion by osteosarcoma cells was assessed using 12-well cell culture chambers containing inserts with 12-μm pores coated with matrigel. The cells were added to the upper chamber at a density of 5 × 104 cells/insert in the presence or absence of meloxicam, and chemotaxis buffer containing 10 μg/ml of fibronectin was placed in the lower chamber. After 24 h of incubation, cells on the upper surface were wiped off with a cotton swab. Cells that had invaded the lower surface were fixed with 70% ethanol, stained with hematoxylin–eosin, and 10 different fields counted under a light microscope at 200× magnification.

TUNEL staining

TUNEL (TdT-terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) staining was used to evaluate the apoptotic effect of meloxicam on MG-63 and LM-8 cells. The cells were seeded on chamber slides (Becton Dickinson Labware, NJ) and allowed to adhere to the bottom of the slides for 12 h. The subconfluent cells were then exposed to medium containing 10, 50 or 100 μM meloxicam, and the medium was changed every 24 h. At various times cell growth was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazolium] bromide] assay (Boeringer Mannheim, Germany).

Assay of matrigel invasion

Invasion by osteosarcoma cells was assessed using 12-well cell culture chambers containing inserts with 12-μm pores coated with matrigel. The cells were added to the upper chamber at a density of 5 × 104 cells/insert in the presence or absence of meloxicam, and chemotaxis buffer containing 10 μg/ml of fibronectin was placed in the lower chamber. After 24 h of incubation, cells on the upper surface were wiped off with a cotton swab. Cells that had invaded the lower surface were fixed with 70% ethanol, stained with hematoxylin–eosin, and 10 different fields counted under a light microscope at a magnification of 40×, and the percentage of positive-staining cells was calculated as the apoptotic index.

Enzyme immunoassay of PGE2 production

To determine the inhibitory effect of meloxicam on PGE2 production by MG-63 cells, the PGE2 concentration in the culture medium was measured by enzyme immunoassay (ELISA). Cells were plated in 6-well plates at a concentration of 1 × 105 cells/well and allowed to attach before adding 10 or 100 μM meloxicam for 24 or 48 h. The culture media were aspirated and stored at −80°C for ELISA assay using a PGE2 Express ELA Kit (Cayman Chemical Company, MI).

Effects of meloxicam on tumor growth and lung metastasis in vivo

Preliminary observations revealed that MG-63 cells could not grow in nude mice. Instead, we used the murine osteosarcoma cell line, LM-8 (a kind gift from Dr A. Uchida). The cultures of LM-8 used in this study were tumorigenic in vivo. We injected subcutaneously into the dorsal flank of 5-week-old male athymic C3H/He mice. One week after inoculation, small tumors were identified. The mice were randomly divided into three groups (n = 7 mice/group); a control group received normal saline, a treatment group received meloxicam, and a control group received normal saline. The tumors were measured twice weekly, and the tumor volumes were calculated using the formula: V = (A × B × C)/2, where A, B and C are the length, width and depth of the tumor, respectively. The mice were sacrificed when the tumor volume reached 1000 mm3, and the tumor weight was measured.
group, and groups treated with 4 and 40 mg/kg meloxicam (13). Meloxicam diluted in PBS was injected daily intraperitoneally from day 7 to day 28 and the same volume of PBS was injected into the control mice. After 3 weeks, all the mice were killed and their tumors and lungs excised. Tumor wet weight and volume were measured (tumor volume was estimated from the formula \( V = L \times W \times H \times \frac{1}{2} \)). The excised lungs were fixed in 5% buffered formalin and subjected to histological analysis. The number of metastatic lesions in the lungs was counted under a microscope at the coronal-midline section.

To determine the effects of meloxicam, especially in vivo, on antitumor activity, expression of vascular endothelial growth factor (VEGF) in lung metastatic lesion was evaluated by immunohistochemistry. Deparaffinized and rehydrated sections were immersed three times in PBS. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in 30% methanol for 30 min and rinsed in PBS. The slides were then soaked in 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. The primary specific anti-human VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, CA), which has been shown to cross-react with murine VEGF, was applied at a dilution of 1:500, and incubated for 1 h at room temperature. After rinsing with PBS, the sections were processed with a HISTFINE KitR (Nichirei, Japan) and diaminobenzidine-containing substrate solution (HISTFINE KitR).

Statistical analysis
All of the quantitative experiments were performed more than three times. The ANOVA and the post-hoc test (Bonferroni-Dunn) were used to assess differences between means. \( P \)-values of <0.05 were considered statistically significant.

Results

**COX-2 mRNA and protein expression in osteosarcoma cell lines**

Because osteosarcomas are a group of heterogeneous tumors, the expression of COX-2 differs between cell lines, and the efficacy of meloxicam is thought to be dependent on the level of COX-2 expression. COX-2 bands were detected at the expected molecular weights following 35 cycles of conventional RT–PCR (Figure 1A). The results of real time RT–PCR analysis showed that expression of COX-2 mRNA was 10.2-fold and 18.9-fold higher in MG-63 cells than in HOS and U2-OS cells, respectively (Figure 1B), whereas the levels of COX-2 protein were 3.1-fold and 6.0-fold higher (Figure 1C).

**Effects of meloxicam on cell proliferation**

As shown in Figure 2A, meloxicam also inhibited the growth of the MG-63 cells. A maximum inhibitory effect of 28.2% was observed after exposure to 100 \( \mu \)M meloxicam for 48 h. The differences in viability between the control cells and the cells exposed to 10, 50 and 100 \( \mu \)M meloxicam were statistically significant (\( P < 0.0001, P < 0.0001 \) and \( P < 0.0001 \), respectively). As there was a statistically significant difference between the effect of 10 and 100 \( \mu \)M meloxicam (\( P = 0.0040 \)), but no significant difference between 50 and 100 \( \mu \)M meloxicam (\( P = 0.091 \)), subsequent experiments were performed with 10 and 100 \( \mu \)M meloxicam. Effects of meloxicam on cell viability of osteosarcoma cell lines, HOS and U2-OS, which express relatively low levels of COX-2, were also determined. Viability of HOS cells was only inhibited by \( \sim 12.5\% \) after treatment of 100 \( \mu \)M meloxicam for 72 h (Figure 2B), and the viability of the U2-OS cell was not affected (Figure 2C).

**Effects of meloxicam on cell invasiveness**

The average (mean \( \pm SD \)) numbers of cells found on the lower membrane, in 10 fields of view, are summarized in Figure 2D. An aliquot of 100 \( \mu \)M meloxicam suppressed the invasiveness of MG-63 cells by 70.4% by 24 h. Thus, although cell numbers of MG-63 fell by at most 28.2% after 24 h of meloxicam treatment, the effect of meloxicam on cell invasiveness was significant. The number of cells found on the lower membrane was significantly lower in the meloxicam-treated groups than in the control group.

**Fig. 1.** COX-2 expression in human osteosarcoma cells. (A) Conventional RT–PCR analysis of COX-2 mRNAs. Lane 1 is a PhiX174 RF DNA HaeIII digest as molecular weight marker. Lanes 2, 3 and 4 are the amplification products for COX-2 (440 bp) from the total RNA of MG-63, U2-OS and HOS cell monolayers, respectively. (B) Semi-quantitative measurement of COX-2 mRNA by real time RT–PCR. Total RNA isolated from these cell monolayers was subjected to real time RT–PCR, and the values were normalized by GAPDH mRNA levels. (C) Semi-quantitative assay of COX-2 protein by western blotting. Cell lysates of monolayers of each cell cultures were subjected to western blot analysis, and the band intensities were normalized by the intensity of \( \beta \)-actin each. Each experiment was performed in triplicate, and bars are mean \( \pm SD \).
treatment (Figure 2A), the inhibitory effect of meloxicam on invasiveness was much greater, suggesting that meloxicam has a direct inhibitory effect on invasiveness of MG-63 cells. Whereas invasiveness of HOS cells, which express relatively low-levels of COX-2, was inhibited by only 10.5% by 100 μM meloxicam. U2-OS cells, which express the quite low levels of COX-2, were not affected in invasiveness by meloxicam at any concentration (Figure 2D).

**TUNEL assay**
To determine the mechanism of the inhibitory effect of meloxicam on osteosarcoma cell numbers, we identified apoptotic cells by TUNEL staining. As shown in Figure 2F, prominent positive stainable cells were observed in a MG-63 cell culture treated with 100 μM meloxicam. The average percentage (mean ± SD) of apoptotic cells exposed to 100 μM meloxicam was significantly increased compared with the control (P < 0.0001), whereas there was no significant increase in apoptotic index upon exposure to 10 μM meloxicam (P = 0.530). In contrast, apoptosis of HOS and U2-OS cells were not induced by meloxicam at any concentration (Figure 2G).

**Expression levels of Bax and Bcl-2 mRNAs and proteins**
To obtain a more accurate quantitative analysis of apoptotic activity observed in MG-63 cell culture, we examined the level of transcripts of Bax and Bcl-2, two apoptosis-related molecules, by real-time RT–PCR. Expression of Bax mRNA in MG-63 cell cultures was significantly increased after 48 h in 100 μM meloxicam (P = 0.024) (Figure 3A), but the drug had...
no significant effect on Bcl-2 mRNA expression (Figure 3B). Whereas expression levels of Bax and Bcl-2 mRNAs in HOS and U2-OS cells was not affected by any concentration of meloxicam (Figure 3A and B). These results suggest that apoptosis is only stimulated by treatment with 100 \( \text{mM} \) meloxicam for 48 h in MG-63 cell culture. This quantitative result is consistent with the analysis of apoptotic cells by TUNEL staining.

To confirm the significant alteration of Bax expression in MG-63 cells, we carried out a western blot analysis of extracts of MG-63 cell cultures incubated with and without meloxicam for 48 h. Bax protein increased significantly only in response to 100 \( \text{mM} \) meloxicam for 48 h (Figure 3C) \((P = 0.0002)\), and Bcl-2 protein showed no change (Figure 3D). These results support the view that meloxicam induces apoptosis of MG-63 cells only at high concentrations (100 \( \text{mM} \)).

**Effects of meloxicam on prostaglandin levels**

Expression levels of PGE\(_2\) after treatment of meloxicam was determined in MG-63 cell culture. The content of PGE\(_2\), a major product of the arachidonic acid pathway, fell by 28.2 and 50.9\%, respectively after 24 h in 10 or 100 \( \text{mM} \) meloxicam. After 48 h, it had fallen by \~65.1 and 68.6\%, respectively (Figure 4A). It is noteworthy that even low concentrations of the drug (10 \( \text{mM} \)) decreased significantly the production of PGE\(_2\) (P < 0.01).

**The effect of exogenous PGE\(_2\) on MG-63 cells treated with meloxicam**

To determine whether the effects of meloxicam are due to inhibition of COX-2 activity, we added PGE\(_2\), a major product of the arachidonic acid pathway, to MG-63 cell culture along with meloxicam. As shown in Figure 4B, meloxicam continued to inhibit MG-63 cell viability in the presence of PGE\(_2\), although the extent of inhibition was reduced (compared with Figure 2A), and PGE\(_2\) rescued the invasiveness of the MG-63 cells at all concentrations of meloxicam (Figure 4C). However, it did not reverse the increase in Bax mRNA after incubation with 100 \( \text{mM} \) meloxicam for 48 h (Figure 4D). The latter observation, together with the fact that PGE\(_2\) does not completely reverse growth inhibition by meloxicam, indicates that meloxicam has some COX-2-independent effect(s) on apoptosis and cell multiplication.

**Effects of meloxicam on osteosarcoma growth and lung metastasis**

Effects of meloxicam on LM-8 cell viability, invasiveness and apoptosis in vitro were determined by MTT assay, matrigel invasion assay and TUNEL assay, respectively, for prerequisite for an in vivo study. We confirmed by conventional RT–PCR that COX-2 is expressed in LM-8 cells, and found, using the MTT assay, that 100 \( \text{mM} \) meloxicam decreases the viability of these cells by 16.8\% at 72 h \((P = 0.0024)\) (Figure 5A). Moreover, 50 and 100 \( \text{mM} \) meloxicam inhibited the invasiveness of LM-8 cells by 32.4 and 95.9\%, respectively \((P = 0.0070 \text{ and } P < 0.0001)\) (Figure 5B). Whereas any concentration of meloxicam could not induce apoptosis in LM-8 cell culture (Figure 5C) in vivo, meloxicam inhibited LM-8 tumor growth significantly at a dose of 40 mg/kg (Figure 6A and B), and the number of metastatic lesions in the lung was also decreased significantly by administration of 40 mg/kg of meloxicam (Figure 6C). No statistically significant differences
Fig. 4. Effects of meloxicam on PGE2 production by MG-63 cells, and the effects of exogenous PGE2 on MG-63 cells in the presence of meloxicam. (A) PGE2 production by MG-63 cells incubated in the presence or absence of 10 or 100 μM meloxicam for 24 or 48 h was measured by EIA. (B) MTT assay. MG-63 cells incubated with 10, 50 or 100 μM meloxicam in the presence of 10 μM of PGE2 for 24, 48 or 72 h were subjected to MTT assay. (C) Matrigel invasion assay. MG-63 cells incubated with 10 or 100 μM meloxicam in the presence of 10 μM of PGE2 were subjected to matrigel invasion assay for 24 h. (D) Semi-quantitative assay of Bax mRNA levels. MG-63 cells incubated with 10, 50 or 100 μM meloxicam in the presence of 10 μM of PGE2 were subjected to real time RT–PCR for Bax. Each experiment was performed in triplicate and bars represent mean ± SD. *Statistically significant difference from control group (P < 0.05). **Statistically significant difference from control group (P < 0.01).

Fig. 5. Antitumor effects of meloxicam on LM-8 cells in vitro. (A) Viability of LM-8 cells incubated with or without 10, 50 or 100 μM meloxicam was evaluated by MTT assay after 24, 48 and 72 h. (B) The invasiveness of LM-8 cells analyzed by the matrigel invasion assay. Cells placed in the upper chamber were incubated with or without 10, 50 or 100 μM meloxicam, and invading cells were counted after 24 h. (C) Apoptotic index of LM-8 cells. Cells incubated with or without 10 or 100 μM meloxicam for 48 h were subjected to TUNEL assay, and percentage of positive stainable cells were calculated. Each experiment was performed in triplicate and bars represent mean ± SD. **Statistically significant difference from control group (P < 0.01).
in body weight were detected. Immunohistochemical analysis for VEGF revealed that 40 mg/kg of meloxicam prominently suppressed the expression of VEGF in lung metastatic lesion compared with control treatment (Figure 6D and E).

Discussion

There is ample evidence for an important role of COX-2 in cancer (1–4), and several groups have shown that selective COX-2 inhibitors have antitumor activity (5,6,8). However, there have been few studies of COX-2 involvement in the formation of malignant bone and soft tissue tumors or of the effects of COX-2 inhibitors on these tumors. In this study, we showed that MG-63 osteosarcoma cells possess remarkably high levels of COX-2, considerably more than two other osteosarcoma cell lines, HOS and U2-OS. In addition, meloxicam, a preferential COX-2 inhibitor, inhibited the growth and invasiveness of MG-63 cells. Meloxicam inhibited PGE2 production by MG-63 cells even at low concentrations, suggesting that COX-2-derived PGE2 may play an important role in the growth and invasiveness of these cells. We found that exogenous PGE2 partially reversed the inhibitory effects of meloxicam on cell growth. The remaining effect could be due to some limitation of the effectiveness of the ‘exogenous’ PGE2 or to a COX-2-independent pathway. The growth of HOS and U2-OS cells, which have much lower levels of COX-2, was much less sensitive to meloxicam; neither cell line was inhibited by 10 or 50 µM meloxicam, and only HOS cells were inhibited by 100 µM meloxicam. These observations suggest that the antitumor effect of low concentrations of meloxicam is mainly due to a COX-2-dependent mechanism and that the effectiveness of this drug depends on the level of COX-2 expression.

The maximum inhibitory effect for cell viability of meloxicam on MG-63 cells was only ~30% after exposure to 100 µM of the drug for 48 h. Higher levels of inhibition have been reported with other COX-2 inhibitors and other malignant tumor cell lines (7,18–20). This could be due to differences in the effectiveness of the various COX-2 inhibitors used or it could be that the MG-63 osteosarcoma cell line is less sensitive to COX-2 inhibitors than other carcinoma cells, such as prostate, colon and lung cancer cells, which might have a different biology from osteosarcoma.

The matrigel invasion assay revealed dose-dependent inhibition of the invasiveness of MG-63 cells by meloxicam. Previous studies have demonstrated that COX-2-derived PGE2 activates CD44 and MMP-2, and stimulates the invasiveness of cultures of lung cancer cells (21). In addition COX-2 was shown to induce MMP-2 expression at the transcriptional level in experiments on the invasiveness of a rhabdomyosarcoma cell line (22). Ours are the first experiments to demonstrate strong inhibition of the invasiveness of an osteosarcoma cell line by meloxicam. Although the mechanism of this inhibition needs to be further investigated, it suggests that meloxicam may have potential as a therapeutic tool.

In contrast to its inhibitory effects on cell viability and invasiveness, only a high concentration (100 µM) of
meloxicam increased Bax transcript and protein levels in MG-63 cell culture, which was in line with apoptotic index determined by TUNEL assay. Transcriptional level of Bcl-2 was not affected by meloxicam in this study. Considering that Bcl-2 is activated by dephosphorylation, determination of Bcl-2 phosphoprotein level should be necessary for more precise analysis. Since PGE2 production by MG-63 cells was substantially decreased by low concentrations of meloxicam, the apoptotic effect of meloxicam cannot be explained by an effect on a COX-2-dependent pathway but must be due to a COX-2 independent-pathway. Our finding that addition of exogenous PGE2 could not reverse the upregulation of Bax mRNA by a high concentration of meloxicam supports this argument. However, the possibility that other prostaglandins may affect the apoptotic pathway and Bax independent apoptotic pathway should be elucidated. The existence of a COX-2-independent pathway would also account for the incomplete restoration of MG-63 proliferation by PGE2. On the other hand, if the apoptotic effect was completely COX-2-independent, the growth of U2-OS cells, which have quite low levels of COX-2, should be inhibited by high concentrations of meloxicam in the same way as MG-63 cells, which is not the case. This suggests that the apoptotic effect of meloxicam may be the result of a complex interaction between COX-2-dependent and independent pathways. Another possible explanation of the different effect of meloxicam on apoptosis in MG-63 and U2-OS cells is that activation of Bax mRNA expression by meloxicam requires a threshold level of COX-2 activity. A previous study of the apoptotic effect of SC236 (a structural analogue of celecoxib) on colon carcinoma cell lines indeed indicated that selective COX-2 inhibitors could induce apoptosis via a COX-2-independent pathway in COX-2-expressing cells (23). However, it should be noted that the difference of the effect of meloxicam on Bax mRNA expression between MG-63 and other cell lines possibly owing to the presence or absence of an unknown modulator which mediates the signal of high concentration of meloxicam. We may also note that since even high concentrations of meloxicam did not affect the proliferation of U2-OS cells, high concentrations of meloxicam seem not to be ‘cytotoxic’.

Many pathways that mediate the apoptotic effect of COX-2 inhibitors have been identified in colon carcinoma cells. Celecoxib may induce apoptosis in part via a COX-2-dependent pathway that causes activation of caspases 3 and 9 together with cytochrome c release, and in part via a COX-2-independent pathway such as downregulation of cyclin A and B1, and upregulation of p21, Waf1 and Kip1 (18). Another COX-2 inhibitor, NS-398, is reported to induce apoptosis via a MEK/ERK pathway (24). Two other NSAIDs, sulindac and ibuprofen, induce Bax and Bcl-XL expression (25) and inhibit PPAR, respectively (26). Various mechanisms have also been proposed for the apoptotic effects of selective COX-2 inhibitors on prostate carcinoma cells. NS-398 causes downregulation of Bcl-2 protein expression (27), celecoxib causes inactivation of Akt (19) and ibuprofen, a non-selective COX inhibitor, blocks the phosphorylation of IκB (28). Cell cycle arrest was induced by celecoxib via a COX-2 dependent pathway in cholangiocarcinoma cells (20), and, in a culture of pancreatic cancer cells, G0/G1 phase cells increased in response to NS-398 (29), whereas cyclin D1 expression was downregulated by rofecoxib (30). Thus the exact pathways by which COX-2 inhibitors induce apoptosis remain controversial. In this study, although we focused on Bax-mediated apoptosis, another evaluation for apoptosis, such as cell cycle analysis, will give further information.

We also demonstrated an effect of high doses of meloxicam on the growth and metastasis of murine osteosarcoma cells in vivo, although whether meloxicam prolong the survival time in LM-8 inoculated mice should be examined. In fact, the inhibition of cell proliferation and invasiveness appeared to be greater in vivo than in vitro. Immunohistochemical analysis demonstrated that meloxicam markedly reduced the expression of VEGF in lung metastatic lesion compared with the control tissues in LM-8 in vivo. One explanation of the enhanced suppressive effects of meloxicam in vivo might be the decreased angiogenesis via inhibited VEGF production. This result is in accordance with previous studies that angiogenesis of tumor is dominantly modulated by COX-2 derived PGE2 (31,32), a selective COX-2 inhibitor that not only induces tumor cell apoptosis but also downregulates tumor VEGF (33). Another group indicated the significant role of host-derived COX-1 and COX-2 in tumor growth that tumors grown in COX-2−/− mice is decreased as well as vascular density compared with wild-type mice (34), suggesting the host cells as well as tumor cells are affected by COX-2 expression. Effects of meloxicam on local stroma or/and VEGF expression probably resulted in enhanced inhibition in LM-8 inoculated in vivo model. Because we were not able to graft MG-63 cells into nude mice, we were unable to assess the suppressive effects of meloxicam on MG-63 growth in vivo. However, since the inhibitory effect of meloxicam on the growth of MG-63 cells was greater than on LM-8 cells, it seems likely that it would also inhibit the growth and metastasis of MG-63 cells in vivo.

In conclusion, the present study provides evidence of an antitumor effect of meloxicam on the human osteosarcoma cell line, MG-63, in vitro, and on the murine osteosarcoma cell line, LM-8, in vivo; in the latter case the drug inhibits not only tumor growth but also lung metastasis. The prognosis for osteosarcoma patients has improved since chemotherapeutic agents were introduced. However, many patients still develop distant metastasis, and new therapeutic tools are needed. Meloxicam is in widespread use as an NSAID against inflammatory diseases; it may also prove to have a therapeutic role in counteracting the tumorigenicity of osteosarcoma. Further studies of its clinical use in osteosarcoma are required.

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