

Enhanced Inhibitory Effect of Meloxicam, A Cyclooxygenase-2 Inhibitor Combined with Cisplatin on the Growth of Human Ovarian Cancers

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at least additive antitumor activity in the treatment of EOC. The inhibitory effect of their combination on growth of EOC suggests a potential to lead a novel therapeutic strategy against EOC.

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Key words: Meloxicam; CDDP; Ovarian cancer; PGE₂; Angiogenesis; Apoptosis

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ABSTRACT

Cyclooxygenase-2 (COX-2) inhibitors are regarded as potentially important for cancer treatment and some reports have demonstrated that combination of COX-2 inhibitors and other anticancer drugs may produce additive or synergistic activity in the treatment of some human cancers. In this study, we investigated the effects of combination of meloxicam, a selective COX-2 inhibitor, and cisplatin (CDDP), a platinum-type drug, on growth of epithelial ovarian cancer (EOC) cells as well as xenografted tumors derived from EOC cells and evaluated the ability of this combining treatment on cell proliferation, apoptosis, prostaglandin E₂ level and angiogenesis. While proliferation of HTOA cells did not change in CDDP alone at a concentration between 0.005-0.5 µg/mL, it was significantly decreased by combination meloxicam and CDDP in a dose-dependent manner of CDDP. Meloxicam alone, CDDP alone or their combination significantly suppressed the growth of OVCAR-3 tumors xenografted subcutaneously and prolonged the survival of mice with malignant ascites derived from DISS cells as compared to control. Combination of meloxicam and CDDP decreased the expression of COX-2, microsomal prostaglandin E synthase and prostaglandin receptor-3 in tumors. Their combination significantly decreased prostaglandin E₂ and vascular endothelial growth factor in serum as well as in ascites, and significantly reduced microvessel density and induced apoptosis in tumors. In conclusion, these results indicate that combination of meloxicam and CDDP may produce

INTRODUCTION

Epithelial ovarian cancer (EOC) represents an insidious disease that typically has progressed to an advanced stage at the time of diagnosis and no reasonably sensitive or specific tests exist to make routine screening cost-effective for early detection or prevention. As such, advances in therapeutic interventions have had little impact on the long-term reductions in deaths attributable to EOC. Patients with ovarian cancer have the highest mortality rate among gynecological malignancies^[1] and an estimated 130,000 deaths per year still occurs worldwide^[2]. Due to the limited success of current therapy, non-toxic modulators of ovarian cancer growth should be developed.

The cyclooxygenase-2 (COX-2) protein is highly expressed in a variety of human cancers including colon, lung and many other solid cancers^[3-6]. COX-2 has been associated with tumor growth, angiogenesis, invasion, and metastasis^[7-9]. Overexpression of COX-2 may increase the resistance of apoptosis in cancer cells^[10,11]. The reduction of the COX-2 enzyme activity or protein expression may inhibit the cell survival and growth in cancer cells. Over the past decade, COX-2 inhibitors have been found to have chemopreventive and antitumor activity and to potential the effects of chemotherapy in variety of tumors^[12-14]. The currently proposed mechanisms of COX inhibitor antitumor activity include: reduction in cell proliferation, induction of apoptosis, and inhibition of angiogenesis^[15,16]. Some evidences prove that COX-2 expression might play an important role in EOC development^[17] and increased COX-2 expression was associated with chemotherapy resistance

and poor outcome in EOC patients^[18]. We proved previously that meloxicam, a selective COX-2 inhibitor produced potent anti-tumor effect against EOC in conjunction with reduction of angiogenesis and induction of apoptosis. Recently, COX-2 inhibitors in combination with other anticancer drugs including doxorubicin, bleomycin, and 5-fluorouracil, have been evaluated for the anticancer activity in human cancers^[19-21]. Selective COX-2 inhibitors enhance the *in vitro* and *in vivo* antitumor effect of anticancer drugs and this effect is associated with concomitant suppressing intratumor PGE₂ levels, altering expression of Bcl-2, Bax, 15-d PGJ₂ and so on.

Cisplatin (cis-diaminedichloroplatinum (II), CDDP), a clinical anticancer drug, is one of the platinum-type agents. It exhibits cytotoxic effect due to decreased proliferation and induction of apoptosis after DNA damage, commonly used in the treatment of ovarian cancer. The combination of a platinum-type drug and paclitaxel is standard therapy for the first-line treatment of women with EOC who require systemic chemotherapy. Recently, cytotoxicity effects of combination of CDDP with selective COX-2 inhibitors have been evaluated by different investigators on some type cancer cell lines and proved that selective cyclooxygenase-2 inhibitors enhanced sensitivity of cancer cells to CDDP mediated cytotoxicity and apoptosis *in vitro* and *in vivo*^[22,23]. Combination of COX-2 inhibitors and oxaliplatin, a third-generation platinum, can increase the growth inhibition and death in human colon cancer cells^[24].

In this study, we investigated the antitumor activity of meloxicam combining with CDDP on growth of EOC *in vitro* and *in vivo* experiments and evaluated the ability of this combining treatment on regulating prostaglandin E₂ levels, apoptosis and angiogenesis.

METHODS

Cell lines and cell culture

OVCAR-3 and HTOA were obtained from the American Type Culture Collection (Rockville, MD) and the RIKEN Cell Bank (Tsukuba, Japan), respectively. OVCAR-3 was derived from a patient with poorly differentiated papillary adenocarcinoma of the ovary. HTOA was established from a well-differentiated human ovarian serous adenocarcinoma. DISS was kindly provided from Dr. Saga (Jichi Medical School, Tochigi, Japan). It was derived from human ovarian serous adenocarcinoma. OVCAR-3 and DISS cell lines grew in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, HTOA cell lines grew in HamF12 medium supplemented with 15% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. All of cell lines were cultured at 37°C in a water-saturated atmosphere with 5% CO₂/95% air. We checked expression of COX-2 protein with a size of 72 KDa by Western blot analysis in three cell lines (Figure 1). The bands of

COX-2 in Western blot of these cells disappeared after preincubation of the antibody with a COX-2 peptide (data not shown).

Cell proliferation assay

To study the effects of meloxicam (Boehringer Ingelheim, Ingelheim, Germany) and CDDP (Nippon Kayaku, Tokyo, Japan) on proliferation of OVCAR-3, HTOA and DISS cells, 100 µL aliquots of cell suspension (5,000 cells/well) in 96-well microplates were incubated with various concentration of meloxicam or CDDP alone and their combination for 72 hour. Viable cell number was estimated by Alamar blue assay and the values were expressed as intensity of fluorescence^[25]. Briefly, 10 µL of Alamar blue working solution (BioSource, Camarillo, CA) was added to each well and the plate was further incubated at 37°C for 3 hour. The fluorescence intensity was measured with excitation at 544 nm and emission at 590 nm using a microplate reader. The reaction was linear in the range of 40-4,000 fluorescence units, corresponding to 5,000-500,000 viable cells/well. Meloxicam was dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich, St. Louis, MO) at a concentration of 100 µM. Drug dilutions were prepared in a culture medium and the DMSO concentration in the wells with the highest drug concentration did not exceed 1%.

Animal experimentation

The animal experiments were conducted in accordance with the Guidelines for Animal Experimentation, Hirosaki University. Eight-week-old female BALB/c nu/nu mice were used in this study. All mice were group-housed in plastic cages with stainless-steel grid tops in an air-conditioned and 12 hour light-dark cycle maintained room in the Institute for Animal Experiments of Hirosaki University and fed with water and food *ad lib*. Mice were monitored for health every 3 day and weighed weekly.

Cancer-bearing mouse model

OVCAR-3 cells (5×10^6 cells) were inoculated subcutaneously in 500 µL of RPMI1640 medium in the back region of the nude mice. All the mice were numbered, housed separately and examined twice weekly for tumor development. The tumor was grown until the longer diameter became 2 mm before starting treatment. Then, the experimental mice were divided into four groups containing 10 mice each (Day 0). Control group received basal diet alone. Meloxicam group was given 162 ppm meloxicam in the diet everyday until the end of the study. CDDP group was administered CDDP at 5 mg/kg intraperitoneally once on Day 0. The combination treatment group was given meloxicam and CDDP essentially in the same way as administered for their respective individual treatment regimens. The tumor dimensions were measured twice weekly using a vernier caliper and tumor volume was calculated using the equation V

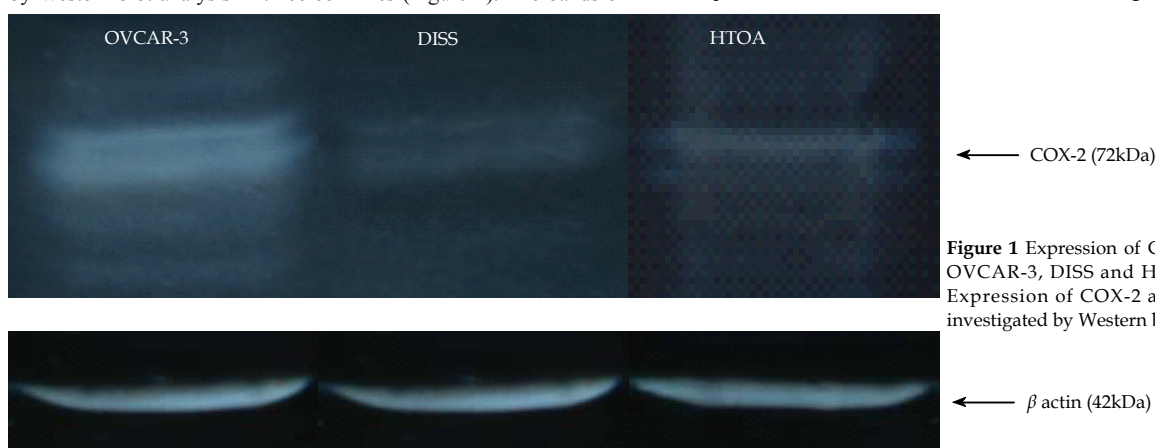


Figure 1 Expression of COX-2 protein in OVCAR-3, DISS and HTOA cell lines. Expression of COX-2 and β -actin was investigated by Western blot analysis.

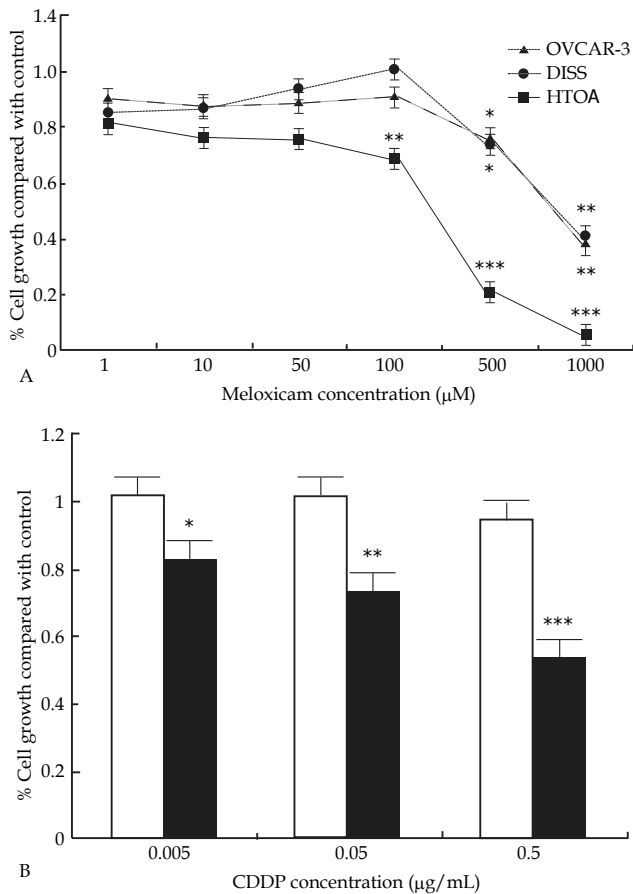


Figure 2 Cell proliferation assay in EOC cells by administration of meloxicam and CDDP. (A) Inhibitory effects of 1-1000 µM meloxicam on proliferation of OVCAR-3, DISS and HTOA cells; (B) Inhibitory effects of combination of meloxicam at a fixed dose of 100 µM and CDDP at a dose of 0.005 to 0.5 µg/mL on proliferation of HTOA cells. White bars represent CDDP alone, and black bars represent combination of meloxicam and CDDP. Data are expressed as the mean±SD of triplicate experiments. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.001$ are significantly different from the control.

(mm^3) = $A \times B^2 / 2$, where A is the largest diameter and B is the smallest diameter^[26]. Serum PGE₂ concentration was determined on Day 7, and the mice were sacrificed on Day 21 to remove the tumor for pathological and biochemical studies.

Cancerous peritonitis mouse model

DISS cells (0.5×10^7 cells) were inoculated into the peritoneal cavity of the nude mice in 500 µL of sterile PBS. It has been reported that the average survival of DISS cell-transplanted mice is about 30 days^[26]. The experimental mice were divided into four groups of each 8 mice. After confirming ascites to be produced on Day 7, the mice were treated in the same way as in the cancer-bearing mouse model. Ascites was aspirated on day 21 for the determination of PGE₂ and VEGF concentrations, and then the survival time for each group was evaluated.

Western blot analysis

Removed tumor tissues were cut into small pieces and homogenized in PIPA buffer in ice. The homogenate was incubated overnight 4°C followed by centrifuging 15,000g for 15 min at 4°C. The supernatants (50 µg protein) were electrophoresed through a 10% SDS-polyacrylamide gel, and blotted as described previously^[27]. The protein concentration was determined using Bradford's method. The blots were probed with the following primary antibodies: COX-2

(IBL, Gunma, Japan) at 2 µg/mL or β-actin (Sigma-Aldrich) at 1:2000. The membranes probed by COX-2 were incubated for 2 hour with anti-rabbit IgG conjugated to horseradish peroxidase and then visualized the immunoblots using diaminobezidine (DAB) (Sigma-Aldrich) as a substrate of peroxidase. β-actin was used as a loading control. The membrane probed by β-actin was incubated for 1 hour with biotinylated anti-mouse immunoglobulin, transferred to avidin-biotin-peroxidase complex reagent (Vector Laboratories, Burlingame, CA), and incubated in this solution for 30 min. DAB (Sigma-Aldrich) was used as a substrate. Quantification of the results was performed by scanning the membrane with Photoshop software (version 5.5, Adobe Systems) followed by densitometry with the public domain software, NIH Image, version 1.62.

Measurement of PGE₂ and VEGF in serum and ascites

PGE₂ concentrations were determined with PGE₂ EIA system (R and D Systems, Minneapolis, MN) according to manufacturer's instructions. VEGF concentrations were determined using an ELISA kit (R and D Systems) as described by Gu *et al.*^[28].

Immunohistochemical analysis and microvessel density

Six-µm sections of formalin-fixed and paraffin-embedded tissue specimens were stained by established method as described previously^[29]. Sections were incubated with antibodies specific for microsomal PGE synthase (mPGES) (Cayman Chemical, Ann Arbor, MI), PGE₂ receptor-3 (EP3) (FabGennix, Frisco, TX), or VEGF (R and D Systems) for 1 hour and CD31 (R and D Systems) overnight. Slides were incubated with biotinylated species-specific appropriate secondary antibodies for 30 min and then exposed to avidin-biotin-peroxidase complex (Vector Laboratories). Sections were treated with 0.02% DAB as a chromogen, and counterstained with hematoxylin. VEGF expression was evaluated according to a scoring method by the positive cell percentage and the staining intensity as reported previously^[29]. Microvessel density was determined as follows. The highly vascularized areas of the tumor stained with an anti-CD31 antibody were identified, and CD31-positive microvessels per 0.75 mm² were counted under high-power field. Single endothelial cells or clusters of endothelial cells, with or without a lumen, were considered to be individual vessels. Microvessel density was expressed as the vessel number/high-power field in sections. Three fields were counted per animal, and the average was taken as the microvessel density of each tumor.

Apoptosis

Apoptosis was measured on tissue sections by the terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick and labeling assay as described by Gavrieli *et al.*^[30] with some modifications. Briefly, 6-µm sections were stripped from proteins by incubation with 10 mg/mL proteinase K for 15 min and immersed in 0.3% H₂O₂ in methanol for 15 min to block the endogenous peroxidase. The sections were then incubated in TdT mixture buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.5, 0.25 mg/mL BSA, 1 mM CoCl₂, 0.01 mM biotin-dUTP, 520 U/mL TdT) at 37°C for 1 hour. After rinsed in PBS, the sections were exposed to avidin-biotin-peroxidase complex at 37°C for 30 min. Cells undergoing apoptosis were visualized with DAB. The numbers of stained tumor cells were counted in three fields at ×200 magnification and the results were averaged.

Statistical analysis

The survival curves were calculated by the Kaplan-Meier method,

and the statistical significance of differences in the cumulative survival curves between the groups was evaluated by logrank test. Other statistical analyses were carried out by Student's *t*-test, Chi square test or Fisher's exact probability test. A result was deemed significant at $P < 0.05$. Data are expressed as means \pm standard deviation (S.D.)

RESULTS

Inhibitory effect of meloxicam, CDDP and their combination on cell growth

To examine the effect of meloxicam and combination of meloxicam and CDDP on *in vitro* cell growth, first we exposed three EOC cell lines to meloxicam at a various concentrations and measured cell proliferation after 72 hours. As shown in figure 2A, meloxicam inhibited the growth of HTOA cells in at concentrations of $\geq 100 \mu\text{M}$ or more. On the other hand, although meloxicam did not inhibit the growth of OVCAR-3 and DISS cells at a concentration of $100 \mu\text{M}$, meloxicam at concentrations of $\geq 500 \mu\text{M}$ or more inhibited the growth of both of cell lines.

Next, we assessed the inhibitory effect of combination of meloxicam and CDDP on proliferation of HTOA cells. A concentration of meloxicam was fixed at $100 \mu\text{M}$ that reduced 20% of proliferation of HTOA cells, and CDDP was administered at various concentrations. While cell growth did not change in CDDP alone at a concentration between $0.005\text{-}0.5 \mu\text{g/mL}$ (Figure 2B, white bars), it was significantly decreased by combination meloxicam and CDDP in a dose-dependent manner of CDDP (Figure 2B, black bars).

Anti-tumor effect of combination of meloxicam and CDDP on cancer-bearing mice and cancerous peritonitis mice

To study the anti-tumor effects of combination of meloxicam and CDDP, we prepared a cancer-bearing mouse model and a cancerous peritonitis mouse model. In the cancer-bearing mice, the combination produced a significantly greater antitumor effect than the control, CDDP, or meloxicam alone treatment. At the end of the experiment, the tumor volumes were 5.94 ± 0.57 , 3.66 ± 0.62 , 4.41 ± 0.54 , and $2.83 \pm 0.62 \text{ cm}^3$ in the control, CDDP alone, meloxicam alone, and their combination, respectively (Table 1). The inhibition rate was 38.4% for CDDP alone, 25.8% for meloxicam alone, and 52.4% for their combination (Table 1). In the cancerous peritonitis model, the survival times were significantly prolonged in CDDP alone, meloxicam alone, and their combination compared with the control ($P < 0.0001$, respectively, figure 3). The combination significantly prolonged the survival time as compared with CDDP alone ($P < 0.05$), whereas no significant difference was found in survival time between meloxicam alone and the combination ($P = 0.13$) (Figure 3). The combination did not result in weight loss or gain of the mice and did not lead to any adverse effects.

Decreased PGE₂ levels in serum and ascites by administration of combination of meloxicam and CDDP

The concentration of PGE₂ in serum of cancer-bearing mice serum

Table 1 Inhibitory effect of combination of meloxicam and CDDP on cancer bearing mice.

Treatment	No. of mice	Volume (cm ³)	Inhibition rate (%)
Control	10	5.94 ± 0.57	
CDDP	10	3.66 ± 0.62 ¹	38.4
Meloxicam	10	4.41 ± 0.54 ²	25.8
Combination	10	2.83 ± 0.62 ^{1,3,4}	52.4

¹ $P < 0.001$, significantly different from the control; ² $P < 0.01$, significantly different from; the control; ³ $P < 0.05$, significantly different from CDDP alone; ⁴ $P < 0.001$, significantly different from meloxicam alone.

was $660 \pm 78 \text{ pg/mL}$ for the control group, whereas it was $595 \pm 69 \text{ pg/mL}$, $133 \pm 31 \text{ pg/mL}$ and $104 \pm 24 \text{ pg/mL}$ for the CDDP alone, meloxicam alone and their combination, respectively (Figure 3, white bars). The concentration of PGE₂ in serum significantly decreased in the combination, compared with the CDDP or meloxicam alone ($p < 0.0001$ and $p < 0.05$, respectively). The mean concentration of PGE₂ in ascites of cancerous peritonitis mice was $709 \pm 256 \text{ pg/mL}$ for the control, whereas it was $505 \pm 120 \text{ pg/mL}$, $199 \pm 36 \text{ pg/mL}$ and $140 \pm 45 \text{ pg/mL}$ for CDDP alone, meloxicam alone, and their combination, respectively (Figure 4, black bars). The concentration of PGE₂ in ascites was significantly lower in their combination than in CDDP or meloxicam alone ($p < 0.0001$ and $p < 0.01$, respectively). Additionally, the concentration of PGE₂ in serum and ascites significantly decreased in meloxicam alone compared with the control ($p < 0.0001$, $p < 0.0001$, respectively).

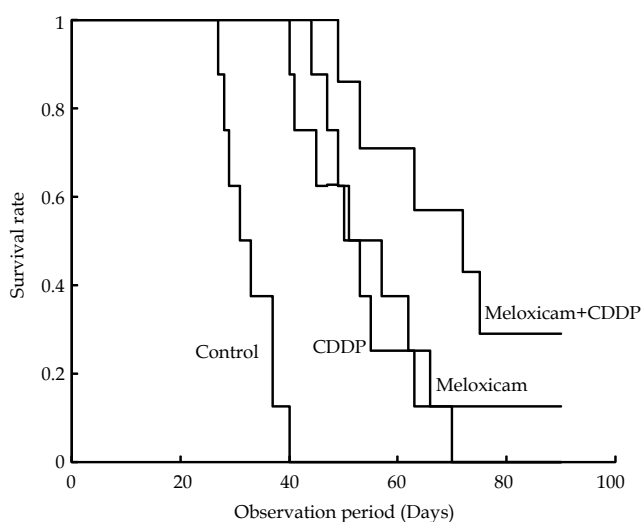


Figure 3 Comparison of survival period in cancerous peritonitis mice. The survival times were significantly prolonged in the CDDP, meloxicam and their combination, compared with the control group ($p < 0.0001$, respectively). The combination significantly prolonged the survival time as compared with CDDP alone ($P < 0.05$), whereas no significant difference was found in survival time between meloxicam alone and the combination ($P = 0.13$).

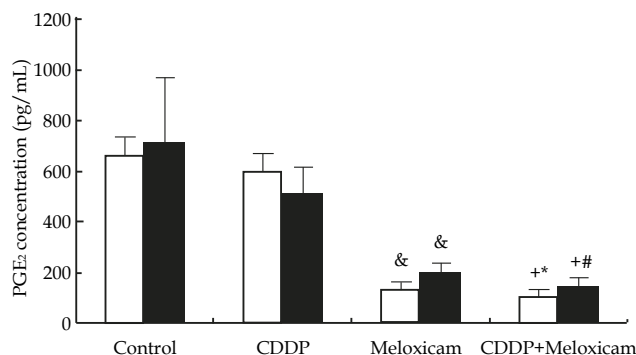


Figure 4 Determination of PGE₂ concentrations in serum (white bars) and ascites (black bars). The concentrations of PGE₂ in serum and ascites significantly decreased in the combination, compared with CDDP alone or meloxicam alone. Additionally, the concentration of PGE₂ in serum and ascites significantly decreased in meloxicam alone compared with the control. There was no significant difference in the concentration of PGE₂ in serum and ascites between the control and CDDP alone. + $P < 0.0001$ versus CDDP alone, * $P < 0.05$ versus meloxicam alone, # $P < 0.01$ versus meloxicam alone, and & $P < 0.0001$ versus the control.

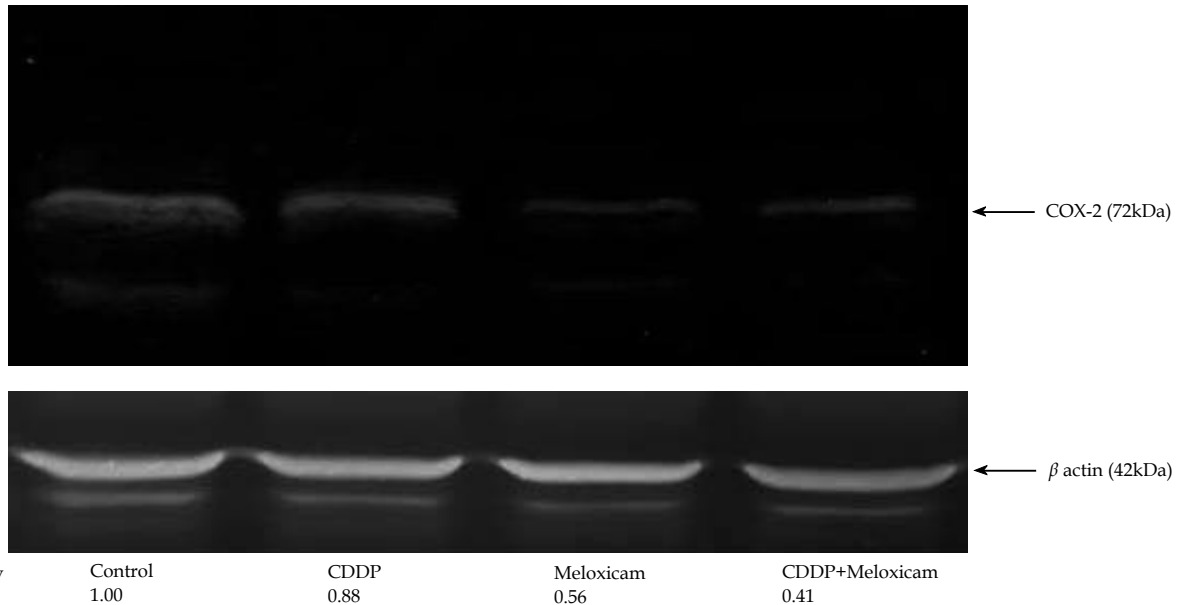


Figure 5 Decreased expression of COX-2 in tumors by meloxicam alone or combination of meloxicam and CDDP. In meloxicam alone-treated tumors and the combination-treated tumors, the level of COX-2 expression was 56% and 41%, compared to the control, respectively. Densitometric values represent the beta-actin corrected means of three blots. Blots shown are one representative experiment of three.

Effect of meloxicam on COX-2 expression in tumors

Expression of COX-2 in tumors was evaluated by Western blotting. This analysis revealed that in CDDP alone-treated tumors, COX-2 expression did not change as compared to control, and that in meloxicam alone-treated tumors and the combination-treated tumors, the level of COX-2 expression was 56% and 41%, compared to the control, respectively (Figure 5).

Altered expression of mPGES and EP3 in tumors by meloxicam or the combination

In order to examine whether expression of mPGES which converts PGH_2 to PGE_2 , and EP3 which is one of the PGE_2 receptors was altered by combination of meloxicam and CDDP, distribution of mPGES and EP3 in tumors was immunohistochemically investigated. We observed stronger staining of mPGES in the cancer cells in specimens obtained from the control tumors (Figure 6A), moderate staining in the cancer cells of tumors from CDDP alone (Figure 6B). There was weak or negligible staining of mPGES in cancer cells of tumors obtained from meloxicam or the combination (Figure 6C and D). Interestingly, in meloxicam alone- and the combination-tumors, mPGES appeared stronger staining of edge cells of cancer nest (Figure 6C and D), whereas in another tumors, its expression distributed evenly in the peripheral and center of the tumor. The staining intensity EP3 paralleled that of mPGES (Figure 6E to H) and its distribution pattern was almost same as that of mPGES.

Inhibitory effect of combination of meloxicam and CDDP on VEGF amount in tumors and ascites

VEGF amount in tumors was evaluated by immunohistochemical staining as shown in figure 7A. The staining score of VEGF was 5.4 ± 0.5 for the control, 4.3 ± 0.7 for CDDP alone, 2.9 ± 0.9 for meloxicam alone, and 2.2 ± 0.5 for the combination (Figure 7B). The VEGF amount significantly decreased in the combination as compared to CDDP alone ($P < 0.001$) or meloxicam alone ($P < 0.05$) alone. VEGF amount in ascites determined by ELISA were 582.3 ± 112.0 for the control, 391.3 ± 44.8 for CDDP alone, 256.3 ± 43.5 for meloxicam alone, and 215.7 ± 40.7 for the combination (Figure 7C). The amount significantly decreased in the combination, compared to CDDP ($P < 0.01$) or meloxicam alone ($P < 0.05$).

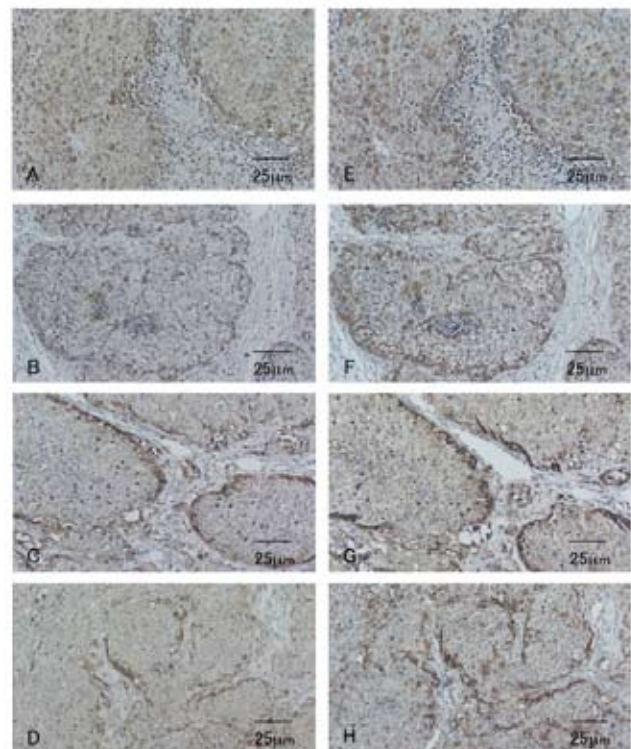


Figure 6 Immunohistochemical staining of OVCAR-3 tumors with anti-mPGES antibody (A-D) and anti-EP3 antibody (E-H). (A) and (E) the control. (B) and (F) CDDP alone. (C) and (G) meloxicam alone. (D) and (H) combination of meloxicam and CDDP. Scale bars show 25 μm .

Reduction of microvessel density and induction of apoptosis in tumors by combination of meloxicam and CDDP

We examined the number of microvessels identified with CD31 in tumors using the immunostaining method. Microvessel density (MVD) (number/ mm^2) was 21.2 ± 4.1 for the control, 19.7 ± 8.0 for CDDP alone, 11.2 ± 2.8 for meloxicam alone, and 7.3 ± 1.5 for the combination, which significantly decreased in the combination as compared with the control, CDDP alone and meloxicam alone ($P < 0.001$, $P < 0.001$, and $P < 0.05$, respectively, Figure 8A). To

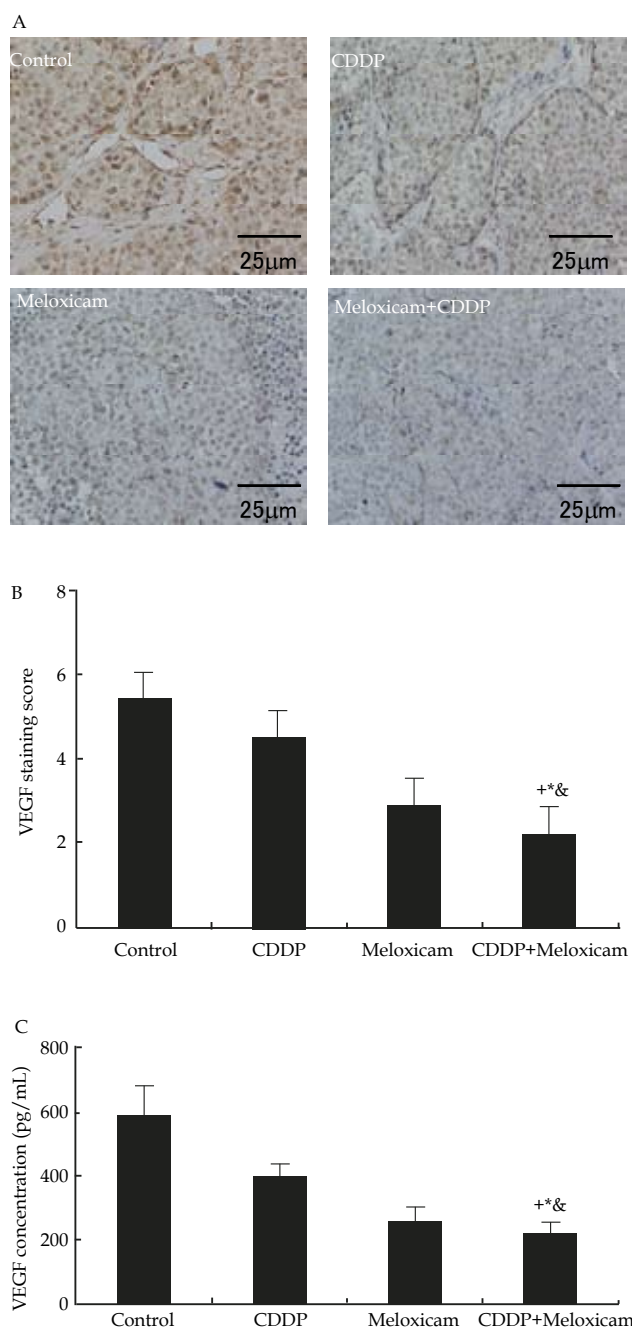


Figure 7 Determination of VEGF amount in tumors and ascites. (A) Immunohistochemical staining of the tumors with anti-VEGF antibody. (B) Semi-quantification of VEGF expression in tumors. The VEGF amount significantly decreased in the combination as compared to the other 3 groups. + $P < 0.001$ versus the control, * $P < 0.001$ versus CDDP alone, & $P < 0.05$ versus meloxicam alone. (C) Determination of VEGF concentration in ascites. The concentration of VEGF in ascites was significant lower in the combination than in the other 3 groups. + $P < 0.001$ versus the control, * $P < 0.001$ versus CDDP alone, & $P < 0.05$ versus meloxicam alone.

evaluate the frequency of apoptosis in tumors, apoptotic cells were stained by the TUNEL method, and TUNEL-positive cells per 0.75 mm² were counted in a high power field. The number of TUNEL-positive cells was 14.2±1.8 for the control, 13.5±2.0 for CDDP alone, 20.3±3.1 for meloxicam alone, and 26.6±4.9 for their combination (Figure 8B). The incidence of apoptotic cells was significantly higher in the combination than in the control, CDDP alone, and meloxicam alone ($P < 0.001$, $P < 0.001$, and $P < 0.02$, respectively, figure 8B).

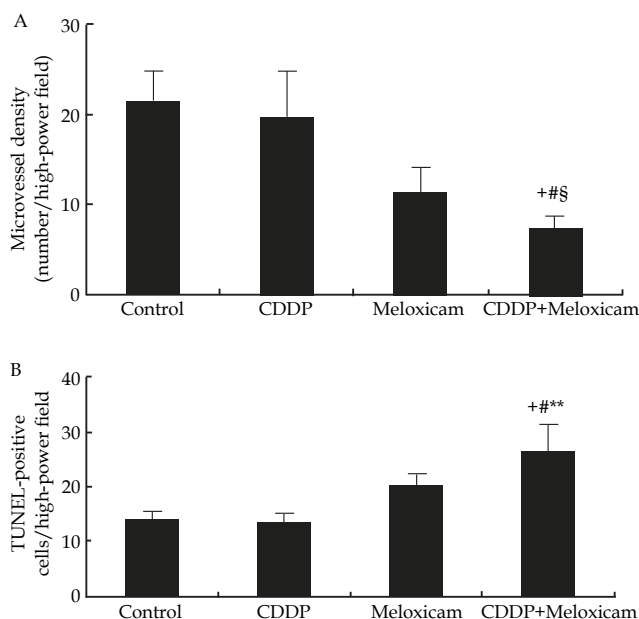


Figure 8 Determination of microvessel density (MVD) and apoptosis in tumors. (A) MVD identified with anti-CD31 antibody in tumors. MVD significantly decreased in the combination as compared with the control, CDDP alone or meloxicam alone. + $P < 0.001$ versus the control, # $P < 0.001$ versus CDDP alone, § $P < 0.05$ versus meloxicam alone; (B) Comparison of the incidence of apoptotic cells in tumors. The incidence of apoptotic cells was significantly higher in the combination than in the control, CDDP alone, and meloxicam. + $P < 0.001$ versus the control, # $P < 0.001$ versus CDDP alone, ** $P < 0.05$ versus meloxicam alone. Results represent mean ± SD.

CONCLUSION

In this study it emerged that combination of meloxicam and CDDP significantly suppressed the growth of the solid tumors and peritoneal carcinomatosa derived from human EOC via reduction of angiogenesis and induction of apoptosis as compared to CDDP alone or meloxicam alone. These results indicate that their combination may produce a great antitumor effect on the growth and progress of EOC. Their combination significantly decreased the PGE₂ concentration in serum and ascites leading to induction of apoptosis and reduction in angiogenesis. This is the first report describing the inhibitory effect of combination of a COX-2 inhibitor and anti-cancer drug on the growth of ovarian cancer.

The COX-2 protein is highly expressed in a variety of human cancers and COX-2 inhibitors have been found to have chemopreventive and antitumor activity^[3-6,12-14]. The antitumor effects of combination of selective COX-2 inhibitors and anticancer drugs have been intensively evaluated by many investigators in recent years^[19-21]. Although combination of a platinum agent and taxans has become the standard chemotherapy for EOC, the addition of COX-2 inhibitor to these agents has not been fully evaluated. Accordingly, we investigated the effects of combination of meloxicam and CDDP on the development of EOC in *in vitro* and *in vivo*.

The present *in vitro* experiment showed that meloxicam enhances the *in vitro* cytotoxicity of CDDP against EOC cells, suggesting the ability of meloxicam to enhance a suppressive effect of CDDP on proliferation of EOC cells (Figure 2). Barnes *et al.*^[23] reported that the combination of CDDP and COX-2 inhibitor increased potentiation of the cytotoxic response in ovarian carcinoma cells because COX-2 inhibitor blocked PGE₂ production. We also showed that although clofibric acid (CA), PPAR α ligand, alone did not affect

proliferation of EOC cells, simultaneous treatment with CDDP and CA significantly suppressed proliferation of the cells compared with that with CDDP alone. CA reduced PGE₂ level in the culture medium, and as a result, the decrease of PGE₂ level might enhance the ability of CDDP to induce apoptosis and anti-angiogenesis^[31]. Thus, because meloxicam significantly decreased PGE₂ concentration as shown in figure 4, enhancement of anti-proliferative and anti-tumor effects of CDDP on EOC might result from reduction in PGE₂ level by meloxicam (Figure 2, 3, and table 1). It has been shown that PGE₂ enhances angiogenesis through the induction of VEGF^[26] and represses apoptosis by maintaining Bcl-2 expression^[32]. Munkarah *et al* also reported that *in vitro* PGE₂ treatment stimulated proliferation of ovarian cancer cells and reduced apoptosis^[33]. PGE₂ production is involved in the ability of cancer cells to invade, metastasize, and grow^[34]. Our data suggest that the pronounced tumor growth inhibition associated with the combination treatment is paralleled by the greatest inhibition of PGE₂ levels indicating the important role of PGE₂ in the tumor development.

Although there was significant difference in reduction of COX-2 expression in tumors between meloxicam alone and combination of meloxicam and CDDP, their combination significantly reduced PGE₂ concentration in serum and ascites as compared to meloxicam alone. So, we investigated whether reduction of PGE₂ concentration is associated with alteration in the expression of other enzymes involved in the PGE₂ biosynthesis and activation. The biosynthesis of PGE₂ from arachidonic acid requires two enzymes that act sequentially. COX is a time-limiting enzyme in the synthesis of PGE₂ by converting arachidonic acid to PGG₂ and subsequently to PGH₂, and mPGES converts COX-derived PGH₂ to PGE₂. The mPGES also plays an important role in releasing PGE₂ from cancer cells^[35] and its enhanced expression is important in tumorigenesis. Cells overexpressing both of COX-2 and mPGES produced more PGE₂, grew faster and exhibited more aberrant morphology than those expressing either COX-2 or mPGES alone^[36]. Moreover, PGE₂ exerts biological function via connecting with its membrane-bound receptors. There are at least four membrane-bound receptors, EP1-4, liganding with PGE₂. Out of those, EP3 is most close to activation of PGE₂ and is overexpressed in human tumor tissues^[37]. Rask *et al* have reported that the expression of COX-2, mPGE synthase and PGE₂ receptor is increased in EOC and that PGE₂-synthesis and signaling are important for malignant transformation and progression of EOC^[38]. The present immunohistochemical staining showed that staining for mPGES and EP3 in the combination-treated tumors was weaker than in the control (Figure 6). These results indicate that alterations in the expression of mPGES and EP3 cause the reduction of PGE₂ level.

Angiogenic factors and anti-apoptotic factors are downstream targets of PGE₂, and as increase in PGE₂ results in up-regulation of VEGF as well as suppression of apoptosis^[39]. Angiogenesis plays a crucial role in tumor development and progression. PGE₂ is a potent inducer of angiogenesis *in vivo* and induces expression of angiogenic regulatory proteins such as VEGF^[40]. Naruse *et al* reported that meloxicam markedly reduced the expression of VEGF in lung metastatic lesion from osteosarcoma compared to the control tissues^[41]. Celecoxib, a COX-2 inhibitor could suppress growth of lung and breast tumors by its potent antiangiogenic activity from reduction of COX-2-derived PGE₂^[42,43]. The present study revealed that combination of meloxicam and CDDP *in vivo* significantly reduced VEGF levels in tumors and the malignant ascites (Figure 7) as well as MVD in tumors (Figure 8A) as compared to meloxicam or CDDP alone, and that their combination significantly produced apoptotic cells in tumors as compared to meloxicam or CDDP alone

(Figure 8B). It is suggested in this study that reduction in VEGF and MVD and induction of apoptosis may be via PGE₂ reduction by combination of meloxicam and CDDP.

In conclusion, the present results suggest that combination of meloxicam and CDDP enhances the *in vitro* and *in vivo* antitumor effect via suppression of PGE₂ concentration and activation. Their combination inhibited the growth of EOC through reducing angiogenesis and inducing apoptosis. This combination may provide a new strategy in human EOC and should be clinical tested.

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