SPANDIDOS PUBLICATIONS

Peroxisome proliferator-activated receptor α and γ ligands inhibit the growth of human ovarian cancer

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Abstract. In this study, we investigated the inhibitory effect of clofibric acid (CA), a ligand for peroxisome proliferator-activated receptor (PPAR)α, and pioglitazone, a ligand for PPARγ, on ovarian cancer in *in vivo* experiments using human ovarian cancer cell lines, and we aimed to elucidate the molecular mechanism of their anticancer effect. The antitumor effects of CA (3,000 ppm in the daily diet), pioglitazone (240 ppm in the daily diet) or the combination were studied in female nu/nu mice, xenografted with subcutaneous OVCAR-3 tumors or with intraperitoneal DISS tumors. The tumor tissues were quantified for expression levels of AP-1, cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) using Western blot analysis or immunohistochemistry. CD-31-stained microvessel density (MVD) was measured in the tumors. The induction of apoptosis was quantified by the TUNEL method. Treatment with CA or pioglitazone significantly suppressed the growth of subcutaneously xenotransplanted OVCAR-3 tumors and prolonged the survival of mice with malignant ascites derived from DISS cells as compared to the control. Combination of both agents enhanced the anticancer effect. Increase of apoptosis and necrosis as well as decrease of VEGF expression and MVD were found in solid OVCAR-3 tumors treated with CA, pioglitazone or the combination. The combination significantly induced apoptotic cells, compared to CA or pioglitazone alone. The combination significantly reduced expression of AP-1, which is a transcriptional regulator of COX-2, and also significantly decreased COX-2 expression in OVCAR-3 tumors compared to the control, CA or pioglitazone alone, although CA or pioglitazone alone decreased them with a marginal significance compared to the control. These findings indicate that the combination of CA and pioglitazone produces a potent antitumor effect on ovarian cancer through reduction of AP-1 expression.

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Introduction

The current management of advanced epithelial ovarian cancer generally includes cytoreductive surgery followed by combination chemotherapy. The combination of paclitaxel with a platinum analogue is the preferred chemotherapy regimen in the treatment of newly diagnosed patients with this disease (1). Although such management induces favorable results in the treatment of ovarian cancer, the long-term survival of ovarian cancer patients still remains unsatisfactory (2), and an estimated 130,000 deaths per year occur from ovarian cancer worldwide (3). Acquired drug tolerance of the ovarian cancer cells is regarded as one of the causes that fail to prolong the survival period of ovarian cancer patients (4). While it is important to elucidate the mechanisms for overcoming drug resistance, new medication, apart from the known chemotherapeutic agents, remains to be developed with a view to improving survival and cure rates in ovarian cancer.

Peroxisome proliferator-activated receptors (PPARs) belonging to the nuclear hormone receptor superfamily exist as three isoforms, PPARα, PPARß/δ, and PPARγ. PPARα plays physiological roles in fatty acid metabolism and catabolism, and PPARγ regulates adipocyte differentiation (5), although the functions of PPARß/δ are not fully known.

Clofibric acid (CA) is a ligand for PPARα and is clinically used for the treatment of hyperlipidemic disorders. CA treatment causes transcriptional increase of acyl CoA oxidase and bifunctional enzyme (enoyl CoA hydratase/L-3-hydroxyacyl CoA dehydrogenase), which are peroxisomal enzymes involved in fatty acid ß-oxidation in the liver of rats via PPARα activation (6,7).

One report demonstrated that a selective ligand for PPARα exerts its protective role against mouse skin tumor promotion (8). It has also more recently emerged that PPARα is up-regulated in endometrial cancer and a PPARα-activating ligand reduces proliferation of endometrial cancer cells (9). These findings raise great interest in the association between PPARα and ovarian malignancy because epidemiological data suggest that dietary fat and cholesterol may increase the risk of ovarian cancer by increasing circulating estrogen levels (10).

Pioglitazone is a PPARγ ligand belonging to the thiazolidinedione (TZD) class of antidiabetic drugs. PPARγ ligands were developed as oral antidiabetic drugs after the discovery that PPARγ activation increases insulin sensitivity and normalizes serum glucose levels (11). Beyond their insulin sensitizing and other metabolic actions, PPARγ ligands exert several other PPARγ-dependent and -independent anticancer effects against a wide variety of neoplastic cells *in vivo* and *in vitro* (12,13). In addition, a moderate anticancer effect of PPARγ ligands with minimal toxicities has been observed in patients with liposarcoma and prostate cancer (13,14).

However, data on PPARα and PPARγ and their ligands in ovarian cancer are scant. In this study, we investigated the effect of CA and pioglitazone on the growth of malignant ovarian tumors in *in vivo* experiments, and we aimed to elucidate the molecular mechanisms of their anticancer effect.

Materials and methods

Cell lines and cell culture. OVCAR-3 was obtained from the American Type Culture Collection (Rockville, MD), and DISS was kindly provided by Dr Y. Saga (Jichi Medical School, Tochigi, Japan). Both cell lines were derived from human epithelial ovarian adenocarcinoma, grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a water-saturated atmosphere of 5% CO_2/95% air. Both cell lines were grown in monolayers.

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, 12 h light-dark cycle maintained room in the Institute for Animal Experiments of Hirosaki University. All animals had access to food and water *ad libitum*.

Cancer-bearing mouse model. OVCAR-3 cells ($0.5x10^7$) in 0.2 ml of RPMI-1640 medium were inoculated subcutaneously in the back region of nude mice. All the mice were numbered, housed separately, and examined three times a week 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 (n=6) (day 0). The control group received a basal diet alone. The CA group was allotted 3,000 ppm CA (Sigma-Aldrich, St. Louis, MO), and the pioglitazone group was allotted 240 ppm pioglitazone (Takeda Pharmaceuticals, Osaka, Japan) in the diet daily until the end of the study. The combination group was given both CA and pioglitazone at the same concentration. The treatment with CA, pioglitazone or the combination did not result in a weight reduction or gain of the mice and caused no adverse effects. The tumor dimensions were measured three times a week using a caliper, and tumor volume was calculated using the equation $V\ (mm^3) = A \times B^2/2$, where A is the largest diameter and B is the smallest diameter (15). The mice were sacrificed on day 21 to remove the tumors for pathological and biochemical studies.

Peritoneal carcinomatosa mouse model. DISS cells ($0.5x10^7$) in 0.5 ml of sterile PBS were inoculated into the peritoneal cavity of nude mice. It was reported that the average survival of DISS cell-transplanted mice is ~30 days. The experimental mice were divided into four groups, n=5. After confirming that ascites were produced on day 7, the mice were treated in the same way as in the cancer-bearing mouse model, and the survival time for each group was evaluated.

Apoptosis. Apoptosis was measured on tissue sections by the terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling assay (TUNEL) as described by Gavrieli *et al* (16) 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% H_20_2 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 $CoCl_2$, 0.01 mM biotin-dUTP, 520 U/ml TdT) at 37˚C for 1 h. After rinsing in PBS, the sections were exposed to avidin-biotin-peroxidase complex (Vecta Laboratories, Burlingame, CA) at 37˚C for 30 min. Cells undergoing apoptosis were visualized with diaminobenzidine (DAB) (Sigma-Aldrich). The numbers of stained tumor cells were counted in three fields at x200 magnification, and the results were averaged.

Immunohistochemical analysis and microvessel density. Sections (6-μm) of formalin-fixed and paraffin-embedded tissue specimens were stained by an established method as described previously (17). Sections were incubated with antibodies specific for VEGF and CD31 (both from R&D Systems, Minneapolis, MN) for 2 h. Slides were incubated with biotinylated species-specific appropriate secondary antibodies for 30 min and then exposed to avidin-biotin-peroxidase complex. Sections were treated with 0.02% DAB as a chromogen and counterstained with hematoxylin. 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 mm^2 were counted under a high-power field. Microvessel density was expressed as the vessel number/high-power field in the sections. Three fields were counted per animal, and the average was taken as the microvessel density of each tumor.

Western blot analysis. Cell lysates (50 μg protein) were prepared from tumor tissues, electrophoresed through a 12.5% SDS-polyacrylamide gel, and blotted as described previously (18). The protein concentration was determined using Bradford's method. The blots were probed with the following diluted antibodies for 2 h: COX-2 (Alpha Diagnostic International, San Antonio, TX) at 1:1000, AP-1/c-Jun (Sigma-Aldrich) at 1:100, and ß-actin (Sigma-Aldrich) at 1:2000. The membranes were then incubated for 1 h with the appropriate biotinylated secondary antibodies, transferred to avidin-biotin-peroxidase complex reagent, and incubated in this solution for 30 min. DAB was used as a substrate. Quantification of the results was performed by scanning the membrane with Photoshop software (version 5.5, Adobe

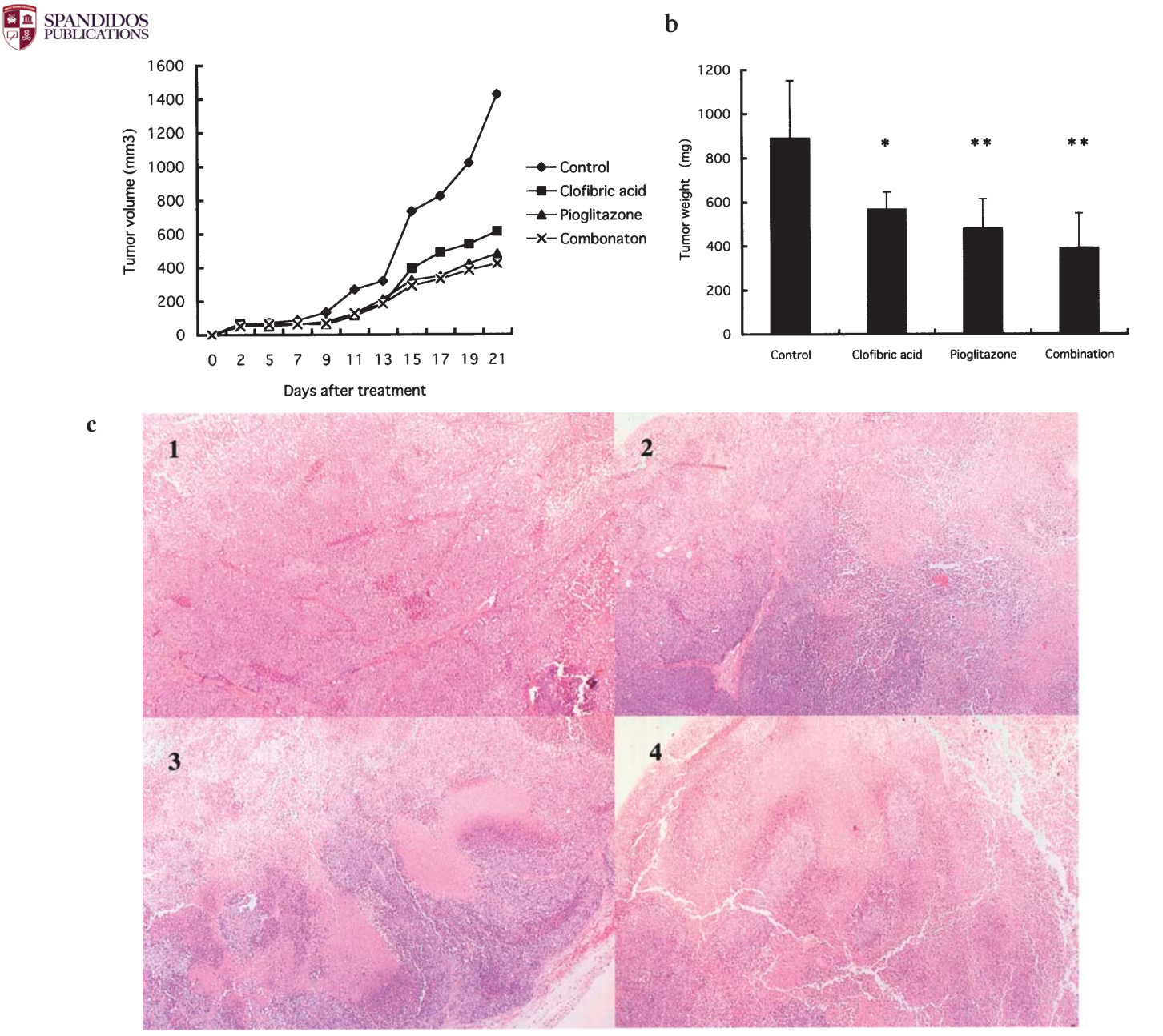

Figure 1. Antitumor effect of clofibric acid (CA), pioglitazone and combination (CA + pioglitazone) on the cancer-bearing mouse model (OVCAR-3). (a) Comparison of tumor growth in cancer-bearing mice. Tumor volume curves at day 9-21 in the CA, pioglitazone and combination groups were found to be significantly lower, compared with the control group (P<0.0001, respectively). (b) The comparison of final tumor weights in cancer-bearing mice. Final tumor weight was significantly smaller in each group compared to the control group. Results represent the means ± SD. *P<0.01 and **P<0.005 versus the control group, respectively. (c) Hematoxylin and eosin staining of tumor specimens. 1, Control group; 2, CA group; 3, pioglitazone group; and 4, combination group. Necrotic lesions were extensively distributed in the CA and pioglitazone groups, and even more extensively in the combination group than in the control. The stained images are one representative of each group including six tumors. Original magnification, x40.

Systems) followed by densitometry with the public domain software, NIH Image, version 1.62.

Statistical analysis. Differences in tumor volume between the groups were analyzed using the non-parametric Mann-Whitney test. 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 the log rank test. Other statistical analyses were carried out by the Student's t-test. A result was deemed significant at P<0.05.

Results

Antitumor effect of CA and pioglitazone in the cancer-bearing mouse and peritoneal carcinomatosa mouse models. In the cancer-bearing mouse model, at the end of the experiment, the tumor volumes were 1,428±350, 614±115, 481±99, and 423±77 mm^3 (expressed as the mean ± SD) in the control, CA, pioglitazone and the combination groups, respectively (Fig. 1a). Tumor volume curves at day 9-21 in the CA, pioglitazone and the combination groups were significantly lower (P<0.0001, respectively), compared with

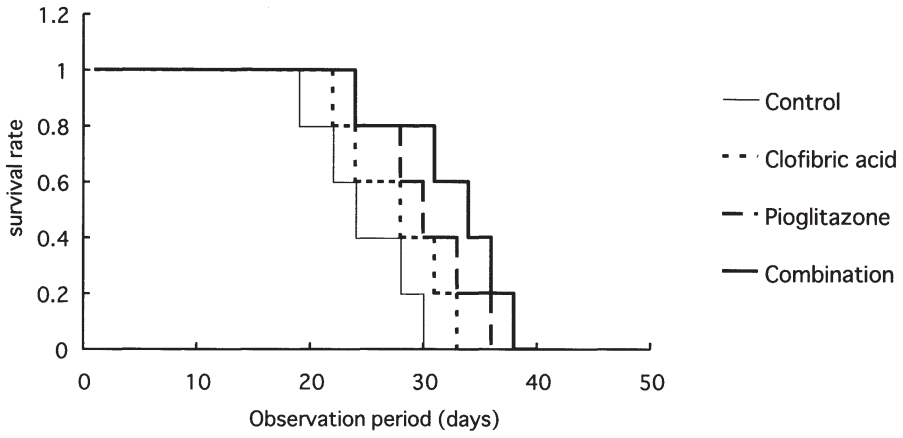

Figure 2. Comparison of the survival periods in the peritoneal carcinomatosa mouse model (DISS). The survival times were significantly prolonged in the combination group, compared with the control group ($P<0.05$). No significant difference in survival time was observed between the control, CA, and pioglitazone groups.

the control group (Fig. 1a). In the comparison of final tumor weights, the tumor inhibition rates were 32% for the CA group, 48% for the pioglitazone group, and 56% for the combination therapy group; tumors being significantly smaller in each treatment group compared to the control group (Fig. 1b). Although there was no significant difference in final tumor weight between the groups of the combination and CA as well as pioglitazone, necrotic lesions were distributed much more extensively in tumors treated with the combination than CA or pioglitazone alone (Fig. 1c). In the peritoneal carcinomatosa mouse model, the survival time was significantly prolonged in the combination group, compared with the control group (Fig. 2). No significant difference in survival time was observed between the control and CA as well as the pioglitazone groups (Fig. 2).

Induction of apoptosis in tumors by CA and pioglitazone administration. To evaluate the extent of apoptosis in tumor tissues in the cancer-bearing mouse model, apoptotic cells were stained by the TUNEL method, and TUNEL-positive cells per 0.75 mm^2 were counted in a high power field. The frequency of the incidence of TUNEL-positive cells (number/0.75 mm^2) was 21.0±2.6 for the control group, 35.7±4.0 for the CA group, 34.0±3.0 for the pioglitazone group and 47.0±2.6 for the combination group (Fig. 3a and b). The incidence of apoptotic cells was significantly higher in the CA, pioglitazone and combination groups than in the control group (Fig. 3b). Moreover, a significant difference was found in the incidence of apoptotic cells between the combination group and CA as well as the pioglitazone groups (Fig. 3b).

Decrease of VEGF expression and microvessel density in tumors by CA and pioglitazone administration. We investigated the distribution of VEGF in tumor tissues using immunohistochemical staining. While VEGF was strongly expressed in cancer cells of the control group, its expression was faint in those of the combination group (Fig. 4a). We also examined the number of microvessels identified with CD31 in tumor tissues using the immunostaining method. Microvessel density (MVD) (number/0.75 mm^2) was 16.0±1.0 for the control group, 9.7±1.5 for the CA group, 8.7±0.6 for the pioglitazone group and 7.7±0.6 for the combination group, which significantly decreased in the CA, pioglitazone and combination groups, compared with the control group (Fig. 4b).

Altered expression of AP-1 and COX-2 in tumors by CA and pioglitazone administration. To examine whether AP-1, which is a transcriptional regulator of several genes, and COX-2 had altered expression in tumors following CA and pioglitazone treatment, we determined expression levels of AP-1 and COX-2 using Western blot analysis. The expression levels of AP-1 decreased to 0.79-fold in the CA group, 0.68-fold in the pioglitazone group, and 0.20-fold in the combination group, compared with the control group (Fig. 5a). The expression levels of COX-2 decreased to 0.74-fold in the CA group, 0.57-fold in the pioglitazone group, and 0.23-fold in the combination group, compared with control group (Fig. 5b). Expression of AP-1 and COX-2 in tumors significantly decreased in the combination group, compared with the control group ($P<0.05$, respectively). The bands in AP-1 and COX-2 Western blots disappeared after preincubation of these antibodies with AP-1 and COX-2 peptide, respectively (data not shown).

Discussion

This study demonstrated that CA and pioglitazone suppress the growth of solid and peritonitis ovarian malignancies derived from human ovarian cancer cells, and the combination of both enhances their antitumor effect in conjunction with reduction in angiogenesis and significant induction of apoptosis.

In spite of a significant interest in the role of PPARα in the pathophysiology of metabolic disorders, only a limited number of studies have reported on PPARα in human malignant diseases, much less in ovarian cancers. Holland *et al* showed that up-regulation of PPARα was one of the transcript changes identified in endometrial cancer using cDNA microarray and that treatment with fenofibrate, a ligand for PPARα, significantly reduced proliferation and increased cell

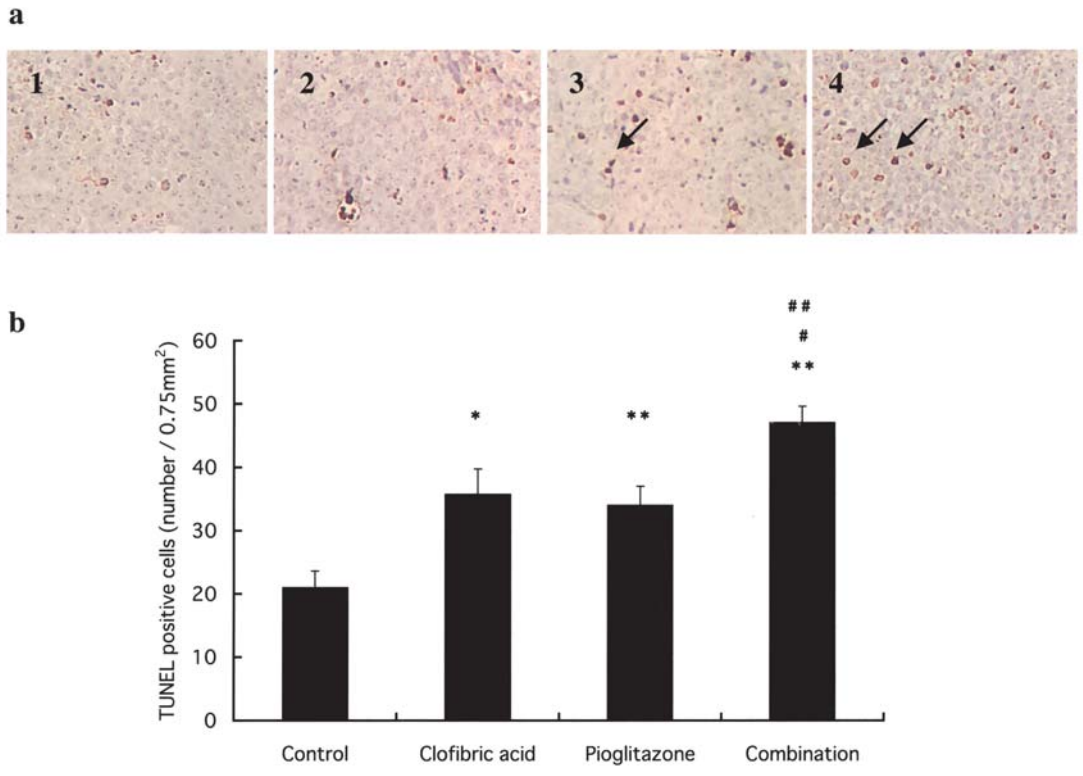

Figure 3. Determination of apoptosis in OVCAR-3 tumors. (a) One representative of each group including six tumors stained by the TUNEL method. 1, control group; 2, CA group; 3, pioglitazone group; and 4, combination group. Arrows show apoptotic cells. Original magnification, x100. (b) The incidence of apoptotic cells was significantly higher in the CA, pioglitazone and combination groups than in the control group. A significant difference was found in the incidence of apoptotic cells between the combination group and CA as well as the pioglitazone groups. Results represent the means ± SD. *P<0.01 and **P<0.005 versus the control group, respectively; $^{\#}$P<0.01 versus CA group and $^{\#\#}$P<0.05 versus the pioglitazone group, respectively.

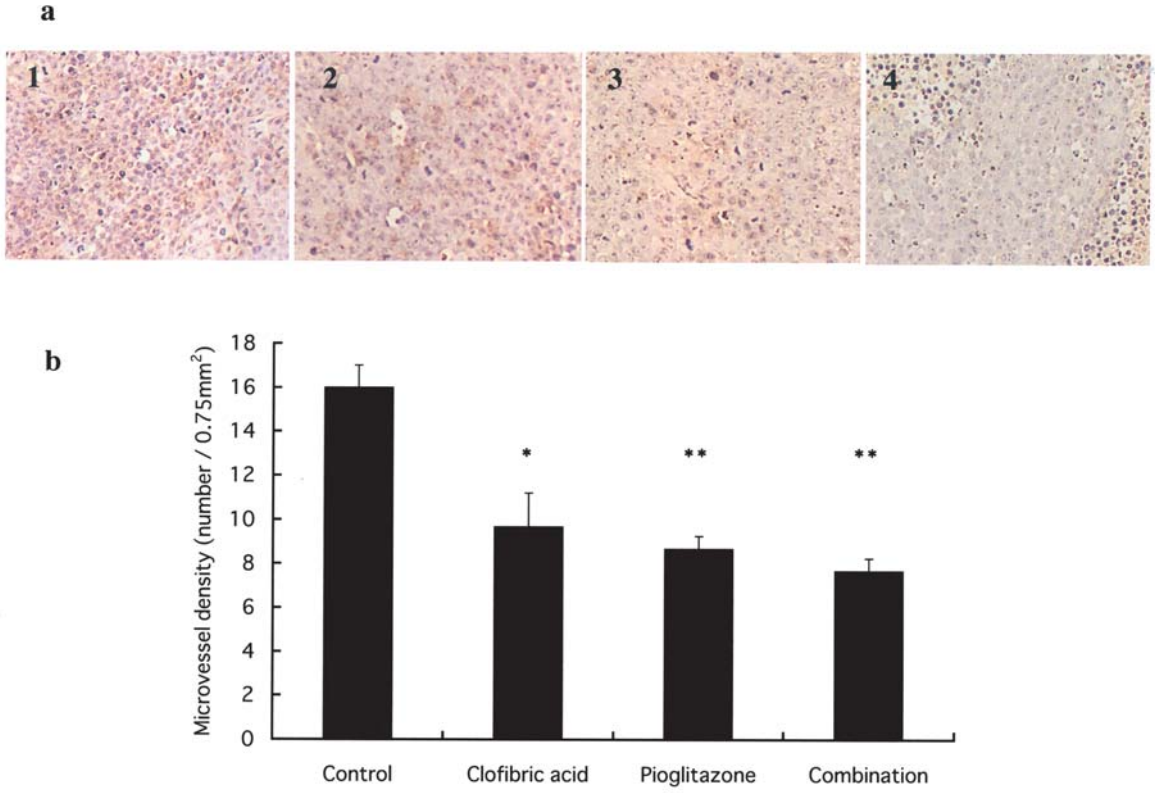

Figure 4. Determination of VEGF and microvessl density (MVD) in OVCAR-3 tumors. (a) Distribution of VEGF in tumors. 1, Control group (strongly expressed); 2, CA group (moderately expressed); 3, pioglitazone group (moderately expressed); and 4, combination group (weakly expressed and surrounded by necrotic tissues). Stained images are one representative of each group including six tumors. Original magnification, x100. (b) MVD identified with anti-CD31 antibody in tumors. MVD significantly decreased in the CA, pioglitazone and combination groups, compared with the control group. Results represent the means ± SD. *P<0.05 and **P<0.005 versus the control group, respectively.

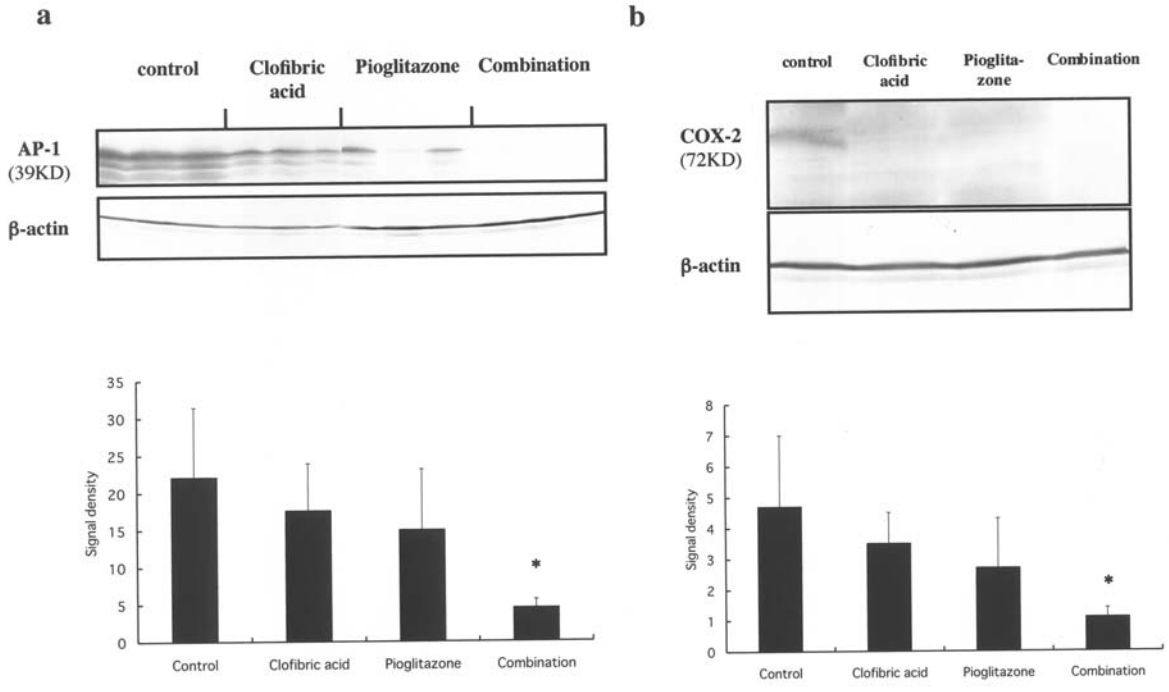

Figure 5. Altered expression of AP-1 and COX-2 in tumors treated with CA, pioglitazone or the combination. (a) AP-1 expression decreased to 0.79-fold in the CA group, to 0.68-fold in the pioglitazone group, and 0.20-fold in the combination group, compared with the control group. (b) COX-2 expression decreased to 0.74-fold in the CA group, to 0.57-fold in the pioglitazone group, to 0.23-fold in the combination therapy group, compared with the control group. *P<0.05 versus the control group. Blots shown are one representative experiment of six.

death in cultured endometrial cancer cells expressing PPARα (9). Braissant *et al* found expression of PPARα in rat ovaries using *in situ* hybridization (19). Toda *et al* reported that fenofibrate inhibited aromatase cytochrome P450 expression in the ovary of mice but did not in PPARα null mice, indicating an important function of PPARα in the ovaries (20). Genes induced by PPARα are primarily associated with ß-oxidation in the cells (21). Although the possibility has been reported that the PPARα ligands reduce the growth of certain types of malignant tumors (9) and prevent carcinogenesis, (8) the mechanism remains unknown.

On the other hand, many studies have shown that PPARγ ligands have antiproliferative activity against many transformed cells and that PPARγ ligands are useful in the setting of adjuvant and chemopreventive treatments of several common tumors. Blunted expression or reduced activity of a variety of inflammatory cytokines and transcription factors such as STAT-1, IL-2, IL-6 NF-κB, AP-1 is likely to slow the growth of transformed cells. Inflammation affects carcinogenesis and cancer progression (22), and PPARγ ligands down-regulate the inflammatory response via several pathways in a variety of cells (23). Furthermore, PPARγ ligands induce apoptosis of cells with decreased expression of the antiapoptotic protein, BCL-2 (24). Han *et al* found that the ability of PPARγ ligands to inhibit COX-2 appears to be mediated predominantly through inhibition of AP-1 protein binding to the CRE site in the COX-2 promoter (25). Sarraf *et al* reported that TZDs decreased the growth of colon cancer cells *in vitro* as well as *in vivo* (12). A few studies have shown that TZDs inhibit proliferation, induce differentiation-like changes and induce apoptosis in breast cancer cell lines both *in vitro* and *in vivo* (12,26). In addition, PPARγ heterozygous knockout mice (PPARγ$^{+/-}$) have an increased susceptibility to develop mammary, ovarian and skin cancer after administration of a carcinogen (27). In addition, some reports suggest that PPARγ ligands exert an anti-cancer effect without PPARγ activation (24,28).

In this study, CA and pioglitazone decreased AP-1 expression in tumors and the combination of these significantly reduced its expression (Fig. 5). Combined treatment of both agents also demonstrated decreased expression of COX-2 and VEGF in tumors. AP-1 is one of the transcriptional regulators of the genes such as COX-2 and VEGF and has been reported to be involved in tumor promotion (29). Many lines of evidence suggest that COX-2 and VEGF are involved in various aspects of tumorigenesis and tumor progression (30). Some reports demonstrated that PPARα and PPARγ ligands negatively regulate AP-1 (31,32). Collectively, all of the results, including ours, strongly suggest that CA and pioglitazone reduce the expression of COX-2 and VEGF via inhibition of AP-1 which is involved in anti-inflammatory and anti-angiogenic effects, and that CA and pioglitazone inhibit ovarian cancer growth. This hypothesis is evidenced by recent reports demonstrating that both PPARα and PPARγ agonists inhibit the transcriptional activation of COX-2 and VEGF in human colorectal cancer cells via inhibition of AP-1 (33).

The present study showed that treatment of CA or pioglitazone significantly induces apoptosis in ovarian cancer cells. Several pathways are involved in enhanced induction of apoptosis by ligands for PPARα and PPARγ (9,24,34). One mechanistic possibility is that a decrease in COX-2 expression by CA and pioglitazone may be involved in the induction of apoptosis. Evidence suggests that overexpression

2 reduces apoptotic susceptibility in colon cancer l prostaglandin E_2 produced by COX-2 represses apoptosis by maintaining Bc1-2 expression (35). Thus, induction of apoptosis in xenografted ovarian tumors may result from the inhibition of COX-2 by CA, pioglitazone or both.

In conclusion, the combination of CA and pioglitazone showed a potent inhibitory effect on ovarian cancer growth in conjunction with significant induction of apoptosis and reduction of angiogenesis via inhibition of AP-1 expression. Some chemicals belonging to the ligands for PPARα or γ are now clinically used just for patients with hyperlipidemia or diabetes. Yet one clinical trial revealed that a PPARγ ligand had an inhibitory activity against prostate cancer with minimal toxicities (13). Moreover, new chemicals with the dual properties of the PPARα and γ ligands have been developed for the treatment of metabolic syndrome (36,37), and the present results further suggest that a dual ligand for PPARα and γ may be a promising agent towards a novel therapeutic strategy against ovarian cancer.

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