Inhibitory effect of carbonyl reductase 1 on ovarian cancer growth via tumor necrosis factor receptor signaling

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Abstract. We investigated the mechanisms of the inhibitory effect of carbonyl reductase 1 (CR1) on ovarian cancer growth mediated by the activation of the tumor necrotic factor receptor (TNFR) pathway. OVCAR-3 and TOV21G cells overexpressing CR1 were constructed by transfecting them with CR1 cDNA by lipofection. CR1-overexpressing and control OVCAR-3 and TOV21G cells were injected subcutaneously into nude mice and the tumor growth was compared between the two groups for 3-4 weeks. The expression of TNFR1 and TNFR2 in tumors was examined immunohistochemically at the end of the experiment. Expression levels of caspase-8 and -3 activated by TNFR1, c-Jun activated by TNFR2, and NF-κB activated by both TNFR1 and TNFR2 were determined using immunohistochemistry and western blot analysis. Tumor growth was significantly suppressed in mice injected with CR1-overexpressing cells. Tumor volume in the CR1 induction group decreased temporarily until 2 weeks. Tumor cell membranes in both CR1 induction and control groups were positive for TNFR1 expression; however, total protein levels did not differ between the two groups. TNFR-2 expression was comparatively weak in both groups. The expression of NF-κB and c-Jun was weaker in the CR1 induction group than in control. In contrast, caspase-8 and -3 expression was higher in the CR1 induction group. Furthermore, the number of apoptotic cells was significantly greater in tumors that appeared after injections of both types of CR1-overexpressing cells than in those of control cancer cells. These results suggest that CR1 induces apoptosis by activating the caspase pathway via binding to TNFR1.

Introduction

Ovarian cancer is the seventh most common cancer in women under the age of 65 years. Epithelial ovarian cancer (EOC) constitutes the majority of ovarian neoplasms (1). It is often not detected until the advanced stages and is the most frequent cause of death among gynecologic cancers (2). Over the last several years, the treatment of ovarian cancer has not appreciably changed. Cytoreductive surgery followed by adjuvant chemotherapy is generally recommended as the primary treatment for advanced EOC. Over 70% of patients respond to chemotherapy initially, but ~50% of advanced cases will relapse (3). Many drugs have been developed so far to treat EOC; however, the improvement in the prognosis of EOC patients is insufficient. Therefore, new clinically useful biomarkers and new targets for EOC treatment need to be identified.

Clofibric acid (CA), a peroxisome proliferator-activated receptor α (PPARα) ligand, which is commonly used for the treatment of hyperlipidemia, may be one of the possible EOC treatments. Our previous in vivo and in vitro studies have demonstrated that CA has an antitumor effect against human ovarian cancer, which is comparable to that of cisplatin (4). We have previously shown that CA treatment induces the expression of carbonyl reductase 1 (CR1), which in turn decreased prostaglandin (PG) E2 levels; the treatment also causes significant induction of apoptosis and profound repression of angiogenesis (4). CR1 is a NADPH-dependent oxidoreductase with a broad specificity for carbonyl compounds, which reduces aldehydes and ketones (5). CR1 is present in a variety of organs, such as the liver, kidney, breast, ovary, and vascular endothelial cells, and its primary function is considered to control fatty acid metabolism (6). Earlier reports have shown that there is a negative relationship between CR1 expression levels and malignant tumor growth (7-10). In order to elucidate antitumor effect of CR1, we transfected mouse ovarian cancer cells with a CR1 cDNA expression vector and investigated the effect of overexpressed CR1 on tumor growth in vivo, and found that tumor growth was significantly inhibited in mice from the CR1 induction group compared to tumor development in animals injected with intact, unmodified cell lines.
Furthermore, the milk fat globule EGF factor 8 (MFG-E8), an ‘eat-me signal’ for phagocytes such as macrophages, was expressed extensively in the cytoplasm of tumor cells and interstitial cells of mice from the CR1 induction group. MFG-E8 is released by apoptotic endothelial cells and induces engulfment of apoptotic cells by macrophage (12). Activated macrophages are the major source of tumor necrosis factor α (TNFα) and TNFα is a key cytokine involved in inflammation, cellular homeostasis, tumor progression and carcinogenesis (13).

Therefore, we focused on TNFα, a potent cytokine, which is produced by many types of cells, including macrophages. TNFα exerts a particularly broad spectrum of whole body and cellular responses, including activation and migration of immune cells, fever, acute phase response, cell proliferation, differentiation, and apoptosis (14). TNFα exerts its effects through two distinct receptors, TNF receptor 1 (TNFR1) and TNFR2 (15). Binding of the inherently trimeric TNFα to TNFR1 and TNFR2 induces receptor trimerization and recruitment of several signaling proteins to the cytoplasmic domains of the receptor (15). TNFR1 transduces apoptotic and anti-inflammatory signals through the recruitment of the Fas-associated death domain protein (FADD) and subsequent recruitment of caspase-8 (16,17). Thereafter, activated caspase-8 initiates a proteolytic cascade that involves other caspasers (caspase-3, -6 and -7) and ultimately induces apoptosis (16,17). TNFR1 also mediates anti-apoptotic and inflammatory responses such as the induction of necrosis factor (NF)-κB through the recruitment of TNF-receptor-associated factor 2 (TRAF2) and receptor-interacting protein 1 (RIP1) (18,19). On the other hand, TNFR2 recruits TRAF2 and TRAF1 to transmit anti-apoptotic and inflammatory signals inducing the expression of NF-κB and c-Jun (20).

In this study, we investigated the mechanism of antitumor effects of CR1 mediated by TNFR1 and TNFR2 signaling.

Materials and methods

Cell lines and cell culture. OVCAR-3 and TOV-21G cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Both OVCAR-3 and TOV-21G cells are derived from human ovarian cancer tissues and are commonly used to produce xenografted solid tumors (10,21-23). The cells were cultured in the RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37˚C in a humidified air containing 5% CO2.

Animal experiments. Animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of 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 room at the Institute for Animal Experiments of Hirosaki University. Mice were kept on a 12/12-h light/dark cycle and given ad libitum access to food and water.

Plasmid DNA preparation. To achieve highly efficient transfection, we used the pCMV6-AC-GFP vector (OriGene Technologies, Rockville, MD, USA) that encodes human CR1, the green fluorescent protein (GFP), and the ampicillin-resistant gene. For amplification, pCMV6-AC-GFP was transformed into E. coli DH5α competent cells by heat shock transformation according to standard laboratory protocols. The transformed bacteria were amplified in LB-ampicillin medium. The plasmids were purified from cultured-transformed bacteria using Maxiprep PureLink HiPure Plasmid Filter DNA Purification kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Plasmid DNA (pDNA) was diluted in sterile water to a concentration of 2 µg/µl.

Transfection. OVCAR-3 and TOV-21G cells were plated into 10-cm well plates and cultured to 70-80% confluence in the RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Then, 24 µg of the coding plasmid was transfected into OVCAR-3 and TOV-21G cells using Lipofectamine (Life Technologies, Rockville, MD, USA) according to the manufacturer's protocol. The vector without CR1 pDNA was used as control. Transfected cells were cultured in the RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) for 48 h. We confirmed the expression of the CR construct using fluorescence microscopy.

Xenograft mouse model. The mice were divided into two groups (n=5 for each group) for each of the two types of cancer cells used. Normal OVCAR-3 cells or OVCAR-3 overexpressing the CR1-DNA (5.0x10^6 cells) were injected subcutaneously in 0.2 ml of RPMI-1640 medium into the back region of nude mice. All mice were numbered, housed separately, and tumor development was examined by measuring 2 diameters twice a week using a caliper. Tumor dimensions were measured twice a week using a caliper. Tumor volume was calculated using the following equation: V (mm^3) = A x B^2/2, where A is the largest diameter and B is the smallest diameter (10). On the third or fourth week of the experiment, animals were sacrificed and tumors were isolated for pathological and biochemical examinations.

Immunohistochemistry. Six-micrometer-thick sections of formalin-fixed and paraffin-embedded tissue specimens were stained by an established method, as described previously (4). Sections were incubated with antibodies specific for TNFR1, TNFR2, caspase-8, caspase-3, NF-κB, and c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. Slides were incubated with appropriate biotinylated species-specific secondary antibodies for 1 h and then exposed to avidin-biotin-peroxidase complex. Sections were treated with 0.02% diaminobenzidine as a chromogen and counterstained with hematoxylin.

Western blot analysis. Cell lysates (25 µg protein) were prepared from tumor tissues, electrophoresed using a 12% precast polyacrylamide gel onto nitrocellulose membranes (Bio-Rad Laboratories). The protein concentration was determined using the Bradford assay. The blots were probed for 2 h with the diluted antibodies against the following proteins: CR1 (Santa Cruz Biotechnology) at 1:500, human caspase-8 (Santa Cruz Biotechnology) at 1:200, caspase-3 (Santa Cruz Biotechnology) at 1:200, NF-κB (Santa Cruz Biotechnology) at 1:500, c-jun (Santa Cruz Biotechnology) at 1:500, and...
β-actin (Sigma-Aldrich, St. Louis, MO, USA) at 1:2,000. The membranes were then incubated for 1 h with the appropriate biotinylated secondary antibodies, and protein bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's procedure.

Statistical analysis. Differences in the number of apoptotic cells between the CR1-overexpressing group and control were evaluated using Student’s t-test. Differences in tumor volume between CR1-overexpressing group and control were also evaluated by Student’s t-test. A result was deemed significant at a P-value <0.05.

Results

CR1 expression levels in normal and CR1-transfected OVAR-3 and TOV21-G cells. CR1 expression levels were compared in normal OVAR-3 and TOV21-G cells and cells transfected with CR1 cDNA. CR1-GFP protein was clearly detected in CR1-DNA transfected cells (Fig. 1A). Western blot analysis clearly showed high expression of CR1 protein in the CR1-DNA transfected cells compared to control (Fig. 1B).

Effects of CR1-transfection on tumor growth in vivo. As shown in Fig. 2A, OVCAR-3 and TOV-21G cells injected in mice formed tumors and showed significant increase in tumor volume by 12 or 18 days after injection (P<0.001), respectively. However, OVCAR-3 and TOV-21G cells transfected with CR1 showed no increase until 12 or 18 days after the injection. Although they showed a slight increase in the volume by the end of the experiment, both size and weight (Fig. 2, P<0.001) were significantly lower than those of normal OVCAR-3 and TOV-21G cells. These results suggested that transfection of CR1 caused an inhibitory effect on tumor growth.

Histological examination on the isolated tumor showed that while malignant cells were densely packed in tumor tissues of control groups (normal OVCAR-3 and TOV-21G cells), malignant cells were sparsely distributed in tumors derived from CR1-overexpressing cell lines (Fig. 3). Necrosis with inflammatory cells was observed in large areas of tumors in CR1 induction groups (Fig. 3).

Effects of CR1-transfection on TNFRI and TNFIR2 expression. Immunohistochemical analysis showed that TNFRI was expressed on cell membranes of tumors from all treatment groups and there were no significant differences in its expression levels between CR1 induction and control groups (Fig. 4). TNFIR2 expression levels were comparatively weak in both groups (Fig. 4).

Effect of CR1-transfection on caspase-8 and -3 expression. Immunohistochemical and western blot analysis showed a high expression of caspase-8 and -3 in tumors formed after injections of CR1-overexpressing OVCAR-3 and TOV-21G cells, respectively. In addition, very weak expression was observed in tumors caused by injections of intact cell lines (Fig. 5).

Effects of CR1-transfection on apoptotic cell number. The number of apoptotic cells per mm² identified with an
anti-caspase-8 antibody (24) was 112.0±19.5 and 77.2±12.8 in tumors caused by CR1-overexpressing OVCAR-3 and TOV-21G cells, respectively. Tumors induced by the injection of control OVCAR-3 and TOV-21G cells exhibited fewer apoptotic cells per mm², that is, 18.0±7.7 and 17.6±1.8, respectively. The differences between control and CR1 induction groups were statistically significant (Fig. 6, P<0.001 each).

Effect of CR1-transfection on NF-κB and c-Jun expression. Western blot analysis showed that both NF-κB and
Figure 4. TNFR1 and TNFR2 were distributed in cell membranes of tumors from all treatment groups and there were no significant differences in their expression levels between CR1 induction and control groups. TNFR2 expression levels were comparatively weak in both groups. Magnification, x200.

Figure 5. Caspase-8 and -3 expression levels in tumors. (A) Immunohistochemistry showed that both caspase-8 and -3 expression levels were higher in tumors caused by CR1-overexpressing OVCAR-3 and TOV-21G cells compared to their expression in tumors induced by control cell lines. Magnification, x200. (B) Western blot analysis showed the same result as immunohistochemistry.
c-Jun expression levels were lower in tumors caused by CR1-overexpressing OVCAR-3 and TOV-21G cells compared to their expression in tumors induced by control cell lines (Fig. 7).

**Discussion**

The results of this study show that subcutaneous injections of CR1-overexpressing OVCAR-3 and TOV-21G cells into the back region of nude mice formed smaller tumors in volume than injection with normal OVCAR-3 and TOV-21G cell lines and were compatible with our previous studies that showed spontaneous regression of malignant ovarian tumors with high expression of CR1 (11) and growth promotion of ovarian cancer cell lines by CR1 suppression (10). Because an increased expression of MFG-E8 in macrophages was also observed in our previous study (11), we speculated apoptosis as a putative mechanism of CR1 function. As shown in Fig. 5, we found that...
expression levels of caspase-8 and -3 were higher in tumors with CR1 overexpressed cell lines, confirming our hypothesis. In addition, expression levels of NF-κB and c-Jun were negatively affected in tumors with CR1 overexpression. NF-κB and c-Jun are known to cooperate to prevent apoptosis induced by TNFα, therefore we were interested to study TNFRs for TNFα with CR1.

TNFR1 is ubiquitously expressed in most tissues, whereas TNFR2 is mainly expressed in immune cells (13). Although both receptors bind TNFα, cellular effects of TNFα in most cell types are predominantly mediated by TNFR1 (25). TNFR1 is an important member of the death receptor family and is capable of inducing apoptotic cell death (26). TNFR2 can also mediate cell death signals, which may be indirectly communicated through TNFR1 (26). As shown in Fig. 4, TNFR1 was expressed to equal levels regardless of indirectly communicated through TNFR1 (26). As shown in Fig. 4, TNFR1 was expressed to equal levels regardless of

In conclusion, the results of this study show that CR1 has anticancer effects by inducing apoptosis through the TNFα system. Of interest, CR1 induced apoptosis of TOV-21G cell lines which are derived from chemo-resistant ovarian clear cell adenocarcinoma (30). Clear cell carcinoma is one of the most frequent of ovarian cancers and has poor prognosis (30), then the results of present study suggests that CR1 might become a new candidate for treatment of clear cell carcinoma. Further studies are required for clinical application of a PPARα ligand or CR1 gene therapy.

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References


