

Indoleamine 2,3-dioxygenase promotes peritoneal dissemination of ovarian cancer through inhibition of natural killer cell function and angiogenesis promotion

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Abstract. The purpose of this study was to clarify the relationship between ovarian cancer peritoneal dissemination and indoleamine 2,3-dioxygenase (IDO) expression, and to explore the possibility of IDO-targeting molecular therapy for ovarian cancer. We transfected an IDO expression vector into the IDO-non-expressing human ovarian cancer cell line OMC-1, and established an IDO-expressing cell line (OMC-1/IDO) to examine the relationship between IDO expression and cancer cell growth *in vitro* and *in vivo*. IDO expression did not influence cancer cell growth and invasion *in vitro*, but promoted tumor growth and peritoneal dissemination *in vivo*. Immunostaining showed that IDO expression inhibited natural killer (NK) cell accumulation in tumors and promoted tumor angiogenesis. In addition, the oral administration of the IDO inhibitor 1-methyl-tryptophan inhibited the growth of OMC-1/IDO-derived subcutaneous tumors in mice. These findings indicate that IDO promotes the peritoneal dissemination of ovarian cancer by inhibiting NK cell accumulation in tumors and promoting angiogenesis, supporting the applicability of IDO-targeting molecular therapy in ovarian cancer.

Introduction

Ovarian cancer affected about 21,000 women in the US in 2008, and about 15,000 died of this cancer. It is the fifth most common cause of cancer death in women (1). Since early ovarian cancer is often asymptomatic, more than half of patients have advanced disease with peritoneal dissemination and ascites at the time of diagnosis (2). Standard treatment for advanced ovarian cancer includes surgery and combination

chemotherapy with a platinum agent and paclitaxel. Ovarian cancer is relatively sensitive to chemotherapy, and many patients achieve remission after combined-modality therapy (3,4). However, the effect is temporary, and more than half of patients experience recurrence and die of the disease. Thus, current therapies have limitations, necessitating the development of new therapies.

The most important mode of ovarian cancer spread is peritoneal dissemination (2). Peritoneal dissemination is established through a multistep process involving cancer cell detachment from the primary tumor, apoptosis avoidance, adhesion to the peritoneum, immunotolerance induction, invasion, and angiogenesis (5,6). However, the factors involved in these steps are largely unknown.

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in the metabolism of the essential amino acid tryptophan, and its activity is detected in various tissues such as the placenta, lung, thymus, small intestine, and spleen (7). IDO was first purified by Shimizu *et al* in 1978 from the rabbit small intestine (8). Subsequent studies reported high levels of IDO activity in pneumonia lesions in a mouse model of influenza virus-induced pneumonia (9), and strong IDO expression in various interferon- γ -stimulated, cultured cells (10). These observations suggested a relationship between IDO expression and immunity.

Cytotoxic T and natural killer (NK) cells play important roles in tumor immunity. These cells are sensitive to tryptophan deficiency, which readily suppresses their function (11). The tryptophan metabolite kynurenine also inhibits NK cell function (12). From these results, it is considered that IDO-expressing tumor cells inhibit the function of cytotoxic T and NK cells through tryptophan deficiency and kynurenine accumulation around the tumor cells, thereby developing immunotolerance. IDO is frequently expressed in various cancers such as prostate, colorectal, pancreatic, and gastric cancers (13). In the field of gynecology, IDO expression has been observed in ovarian cancer and uterine cervical and endometrial cancers (13), and associations between its expression and the prognosis of ovarian and uterine cancers have been reported (14,15).

In this study, we aimed to clarify the relationship between IDO expression and ovarian cancer progression, particularly

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peritoneal dissemination, and to develop an IDO-targeted molecular therapy to inhibit the peritoneal dissemination of ovarian cancer.

Materials and methods

Cell culture. The human ovarian cancer cell line OMC-1 (16) used in this study was kindly provided by Dr O. Hayakawa, Department of Obstetrics and Gynecology, Sapporo Medical University. Cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) containing 10% inactivated fetal calf serum (JRH, Lenexa, KS), 100 U/ml of penicillin, and 100 μ g/ml of streptomycin (Gibco, Grand Island, NY) at 37°C in a 5% CO₂ atmosphere for no longer than eight weeks after recovery from frozen stocks.

The NK cell line NK92 was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in α minimum essential medium (Sigma) supplemented with 100 U/ml of human interleukin-2 (Wako, Osaka, Japan), 12.5% inactivated fetal calf serum (Biological Industries, Haemek, Israel), and 12.5% horse serum (Gibco) at 37°C in a 5% CO₂ atmosphere.

Antibodies. Anti-human IDO monoclonal antibody was prepared as previously reported (10). Anti-human β -actin antibody (Sigma), anti-mouse CD49b antibody (R&D Systems, Minneapolis, MN), and anti-mouse von Willebrand factor (vWF) antibody (Millipore, Billerica, MA) were obtained from commercial sources, and used in this study.

Establishment of an IDO-expressing cell line. The human IDO expression vector pcDNA3.1-IDO (17) or pcDNA3.1 (Invitrogen, Carlsbad, CA) was used to transfect OMC-1 cells employing PolyFect Transfection Reagent (Qiagen, Hilden, Germany) according to the instruction manual. Subsequently, the cells were cultured in medium containing 200 μ g/ml of G418 (Sigma) for 4 weeks, and a resistant cell line was obtained.

Western blot analysis. Protein (20 μ g) extracted from a homogenate of cultured cells was mixed with 2x SDS-PAGE sample buffer [120 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 10% 2-mercaptoethanol]. The mixture was heated at 100°C for 5 min, electrophoresed on a 0.1% SDS-12.5% polyacrylamide gel, and proteins were blotted onto a polyfluorovinylidene membrane. The blots were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) at room temperature for 1 h, and reacted with a 1:1,000 dilution of anti-human IDO monoclonal antibody or a 1:200 dilution of anti-human β -actin polyclonal antibody for 1 h at room temperature. The membrane was washed with phosphate-buffered saline (PBS)-Tween-20 3 times, reacted with horseradish peroxidase-conjugated secondary anti-mouse antibody (Nichirei, Tokyo, Japan) or anti-rabbit antibody (Nichirei), and signals were detected by chemiluminescence (ECL kit, Amersham Biosciences, Piscataway, NJ) on X-ray film.

IDO activity measurement. Cultured cells were treated with trypsin, washed with PBS, resuspended in Tris-HCl (pH 6.8),

disrupted with an ultrasonicator on ice, and then centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was collected and mixed with an equal volume of the reaction buffer [50 mM potassium phosphate buffer (pH 6.5), 100 μ g/ml of catalase, 10 μ M methylene blue, 20 mM ascorbic acid, and 200 μ M L-tryptophan]. The mixture was incubated at 37°C for 60 min, and then for 30 min at 50°C after the addition of 30% trichloroacetic acid, followed by centrifugation at 15,000 rpm for 10 min at room temperature. The supernatant was collected, and the kynurenine concentration was determined on 96-well plates by high-performance liquid chromatography.

Cell growth in vitro. Tumor cells (1×10^5 cells) were seeded into each well of a 6-well plate, and cultured in RPMI-1640 medium containing 10% fetal calf serum. Starting at 96 h after seeding, cells were harvested using 0.05% trypsin-EDTA every 24 h, and counted with a hemocytometer to draw a growth curve.

Cell invasion in vitro. Tumor cells (1×10^4 /well) were placed in a Matrigel invasion chamber (Becton-Dickinson, Bedford, MA), cells that invaded through the Matrigel membrane were stained with crystal violet 24 h later, and the number of cells in five high-power fields was counted.

Cytotoxic activity of NK cells against tumor cells in vitro. Tumor cells were labeled with 100 μ Ci of Na₂⁵¹CrO₄ (American Radiolabeled Chemicals, St. Louis, MO) for 1 h at 37°C in a 5% CO₂ atmosphere, and were subjected to experiments after washing twice. NK92 cells (1.25×10^6) were co-cultured with labeled tumor cells (5×10^4) in 200 μ l of RPMI-1640 medium containing 10% inactivated fetal calf serum for 4 h at 37°C in a 5% CO₂ atmosphere. After centrifugation, the cells were collected, and the γ rays emitted from ⁵¹Cr released from lethally injured tumor cells were measured with a γ counter. Percent cytolysis was calculated as follows: cytolysis (%) = [(measured amount of release) - (amount of spontaneous release)]/[(amount of maximum release) - (amount of spontaneous release)] x 100.

Experimental animals. Four- to six-week-old female BALB/c nude mice (Japan Clea Laboratories, Tokyo, Japan) were used for experiments. All animal experiments were performed in accordance with the guidelines for Animal Experimentation of Jichi Medical University.

Subcutaneous tumor growth in vivo. Tumor cells (3×10^6) were inoculated subcutaneously into the back of mice to induce tumor growth. The tumor volume [(long diameter) x (short diameter)² x 1/2] was measured once a week to draw a tumor growth curve.

Peritoneal dissemination in vivo. Tumor cells (3×10^6) were injected intraperitoneally into nude mice, and the mice were observed until death. A survival curve was constructed using the Kaplan-Meier method. The mice were checked for survival twice a day. Their body weight and abdominal circumference were measured, and the difference between their measurements was obtained.

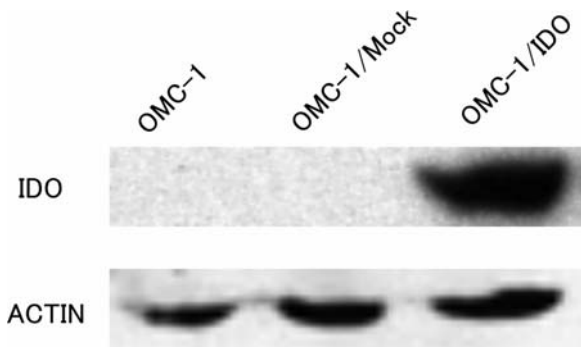


Figure 1. Western blot analysis using monoclonal antibody. IDO expression vector-transfected cells (OMC-1/IDO) alone showed evident IDO protein expression.

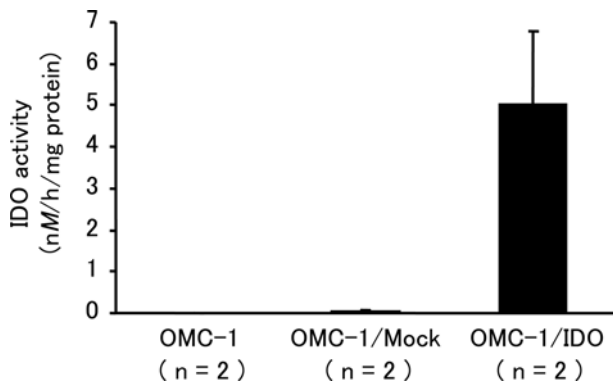


Figure 2. IDO activity evaluated by measuring the kynurenine concentration in each cell culture extract using HPLC. OMC-1/IDO cells alone showed strong IDO activity.

Inhibition of *in vivo* tumor growth in IDO-expressing cells by the oral administration of the IDO inhibitor 1-methyltryptophan (1-MT). IDO-expressing cells (3×10^6) were injected subcutaneously into the back of nude mice, and, immediately thereafter, the mice were allowed free access to sterilized water with or without 1 mg/ml of 1-MT (Sigma). The tumor volume was measured once a week to draw a tumor growth curve.

Immunohistochemical staining. At 2 weeks after tumor cell inoculation, mice were sacrificed under diethyl ether anesthesia, and the tumor was removed. After formalin fixation, paraffin sections were prepared, deparaffinized, and treated with hydrogen peroxide for 30 min to block endogenous peroxidase. Then, the sections were reacted with a 1:10 dilution (5 μ g/ml) of primary antibody for 16 h at room temperature, and, after 3 washes with PBS, were incubated with enzyme-conjugated streptavidin for 30 min. The sections were washed with PBS 3 times, and color was developed using the DAB method. The number of stained NK cells was counted under high-power magnification (x400).

vWF staining was performed using a Blood Vessel Staining kit (Millipore) according to the instruction manual. The number of stained blood vessels was counted under high power magnification (x400).

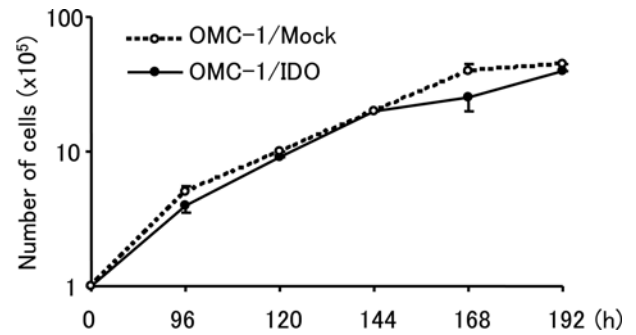


Figure 3. Cell growth curves of OMC-1/IDO and OMC-1/Mock (control) cells. The doubling times of these two groups of cell were about 20 h, showing no intergroup difference.

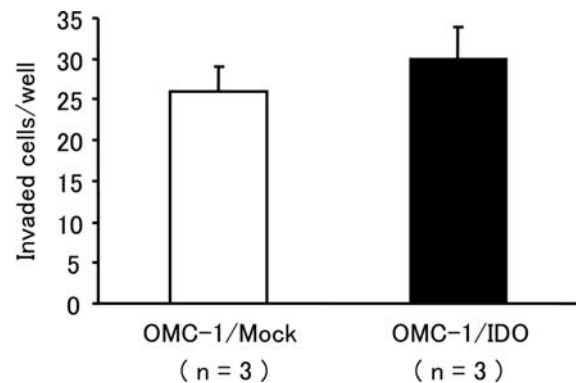


Figure 4. The numbers of invading OMC-1/IDO and control cells. The numbers of invading OMC-1/IDO and control cells were 30 ± 4 /well and 26 ± 3 /well, respectively, showing no significant difference.

Statistical analysis. Except for the comparison of survival curves, the test of significance between the two groups was performed using Student's t-test. The log-rank test was used to compare survival curves between the two groups. A $P < 0.05$ was considered significant.

Results

Establishment of an IDO-expressing cell line. Fig. 1 shows the results of Western blot analysis of the IDO expression vector- or control vector-transfected ovarian cell line OMC-1. Only the IDO expression vector-transfected cells (OMC-1/IDO) showed evident IDO expression. Fig. 2 shows the levels of IDO activity in these cell lines. In concert with the results of Western blot analysis, only OMC-1/IDO cells showed strong IDO activity (5.0 ± 1.8 nM/h/mg protein). Thus, the IDO-expressing cell line OMC-1/IDO was successfully established.

***In vitro* cell growth curve.** Fig. 3 shows the cell growth curves of OMC-1/IDO and OMC-1/Mock (control) cells. The doubling times of these two groups of cells were about 20 h, showing no intergroup difference. Thus, the IDO expression did not influence the growth of OMC-1 cells *in vitro*.

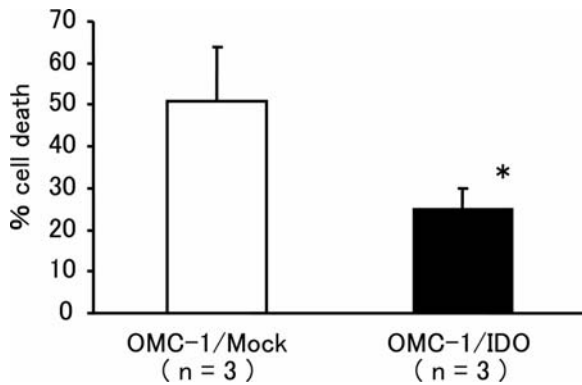


Figure 5. The percent cytotoxicity of tumor cells damaged by co-culturing with NK cells. The percent cytotoxicity (25.1±4.7%) of OMC-1/IDO cells was significantly lower than that (51.0±13.0%) of control cells. *P<0.05. Mean ± SD.

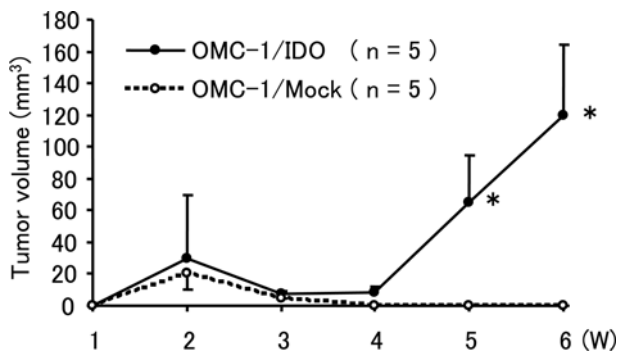


Figure 6. Subcutaneous tumor growth curves of OMC-1/IDO and control cells. Both groups of cell formed small nodules in the second week after inoculation. Subsequently, the tumors in the OMC-1/IDO group enlarged, whereas those in the control group disappeared. *P<0.05. Mean ± SD.

Cell invasion ability in vitro. As shown in Fig. 4, the numbers of invading OMC-1/IDO and control cells were 30±4 and 26±3/well, respectively, showing no difference. In other words, the IDO expression did not influence the invasive capacity of OMC-1 cells *in vitro*.

Cytotoxic activity of NK cells against tumor cells in vitro. The percent cytotoxicity of tumor cells co-cultured with NK cells is shown in Fig. 5. The percent cytotoxicity (25.1±4.7%) of OMC-1/IDO cells was significantly lower than that (51.0±13.0%) of control cells (P<0.05). That is, the IDO expression inhibited the cytotoxic activity of NK cells against tumor cells.

Tumor growth in vivo. The subcutaneous tumor growth curves of OMC-1/IDO and control cells are shown in Fig. 6. Cells in both groups formed small nodules in the second week after inoculation. Subsequently, the tumors in the OMC-1/IDO group enlarged, whereas those in the control group disappeared. Thus, the IDO expression promoted OMC-1/IDO tumor growth *in vivo*.

The number of NK cells in the tumor stroma. Fig. 7 shows the results of the immunostaining of NK cells (black arrow-head) accumulating in the stroma of OMC-1/IDO and control subcutaneous tumors. Fig. 8 presents the number of NK cells per high-power field. The number of NK cells (7±4) that accumulated in the OMC-1/IDO tumors was significantly lower than that (32±8) in the control tumors (P<0.05). Thus, the IDO expression inhibited NK cell accumulation around the tumor.

Tumor angiogenesis. Fig. 9 shows the results of immunostaining of newly formed, vWF-positive blood vessels in the OMC-1/IDO and control subcutaneous tumors. Fig. 10

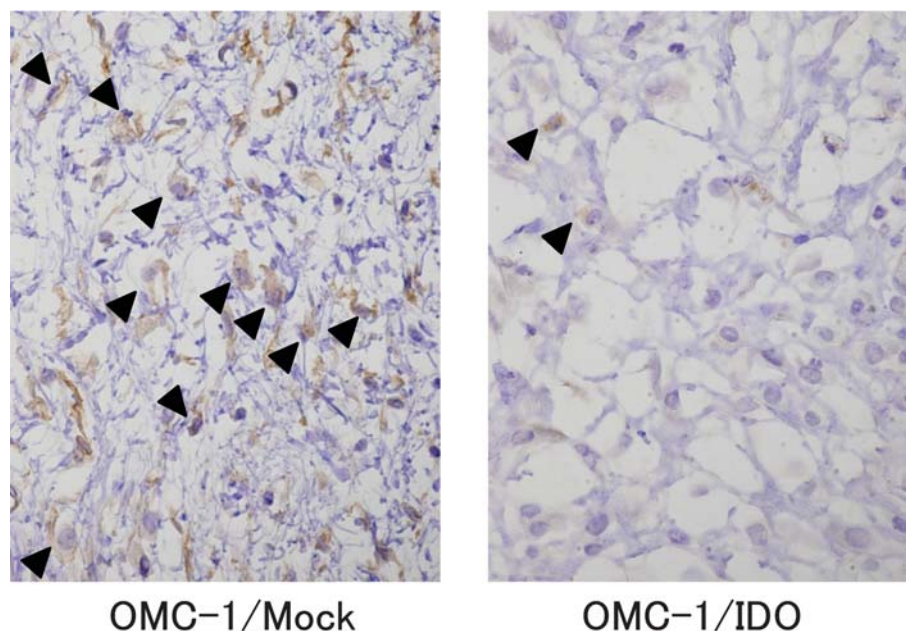


Figure 7. Immunostaining of OMC-1/IDO and control subcutaneous tumors using anti-mouse CD49b antibody. The black arrowhead indicates NK cells accumulating in the tumor stroma.

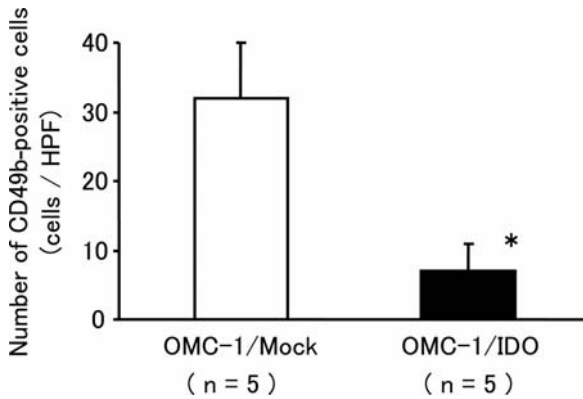


Figure 8. The number of NK cells per high-power field. The number of NK cells (7 ± 4) that accumulated in the OMC-1/IDO tumors was significantly lower than that (32 ± 8) in the control tumors. * $P < 0.05$. Mean \pm SD.

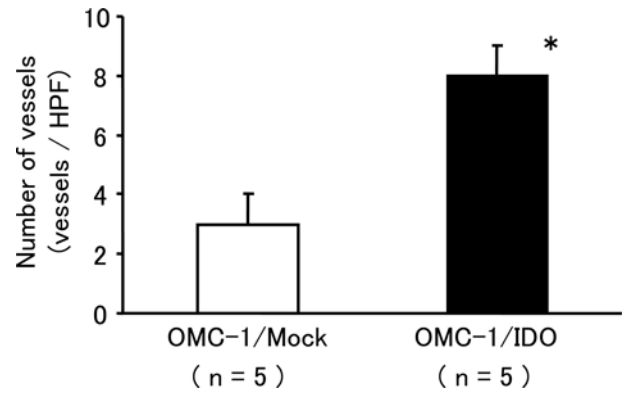


Figure 10. The number of newly formed blood vessels. The number (8 ± 1) of newly formed blood vessels in the OMC-1/IDO tumors was significantly greater than that (3 ± 1) in the control tumors. * $P < 0.05$. Mean \pm SD.

presents the number of newly formed blood vessels per high-power field. The number (8 ± 1) of newly formed blood vessels in the OMC-1/IDO tumors was significantly greater than that (3 ± 1) in the control tumors ($P < 0.05$), indicating that the IDO expression promotes tumor angiogenesis.

Peritoneal dissemination in vivo. Fig. 11A-C show the appearance of mice, their ascites, and peritoneal dissemination, respectively, at 4 weeks after intraperitoneal tumor cell inoculation. Fig. 12 and 13 present the changes in the abdominal circumference and body weight of mice, respectively. Mice with intraperitoneally injected control cells showed no abnormal changes, whereas those receiving the intraperitoneal injection of OMC-1/IDO cells had bloody ascites and marked peritoneal dissemination at 4 weeks after inoculation. The

increases in the abdominal circumference and body weight (19 ± 6 mm and 4.3 ± 0.6 g, respectively) of OMC-1/IDO cell-inoculated mice were significantly greater than those (6 ± 2 mm and 2.7 ± 1.6 g, respectively) of control cell-inoculated mice ($P < 0.05$ each). Fig. 14 shows the survival curves of intraperitoneally inoculated mice. All control cell-inoculated mice survived longer than 70 days, whereas all OMC-1/IDO cell-inoculated mice died of peritoneal dissemination with ascites within 40 days after inoculation ($P < 0.05$). Thus, the IDO expression promoted peritoneal dissemination and ascites in OMC-1-inoculated mice.

Inhibition of tumor growth in IDO-expressing cells by oral 1-MT administration. Fig. 15 shows the *in vivo* subcutaneous tumor growth curve of OMC-1/IDO cells. The non-1-MT-

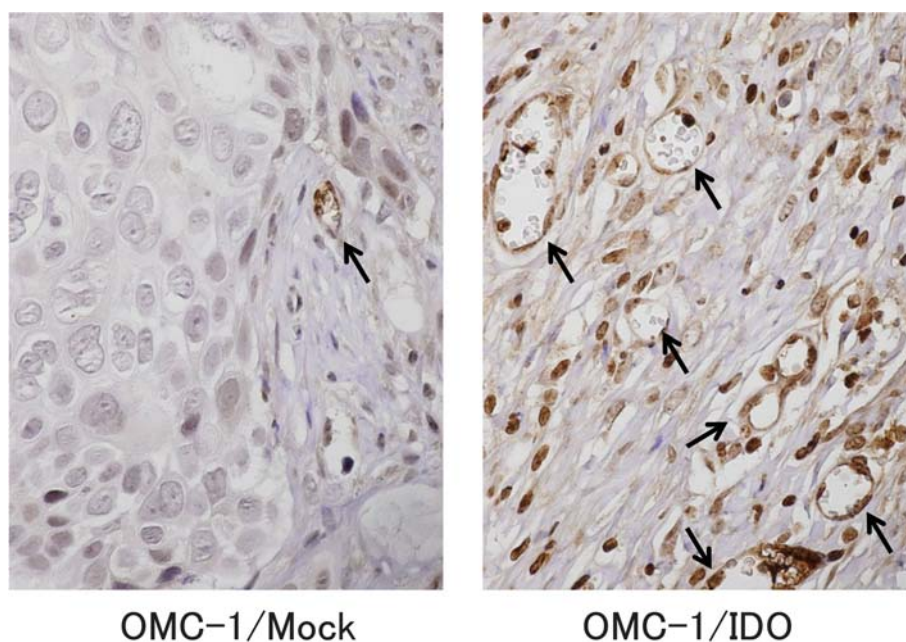


Figure 9. Immunostaining of OMC-1/IDO and control subcutaneous tumors using anti-mouse vWF polyclonal antibody. The black arrow indicates newly formed blood vessels.

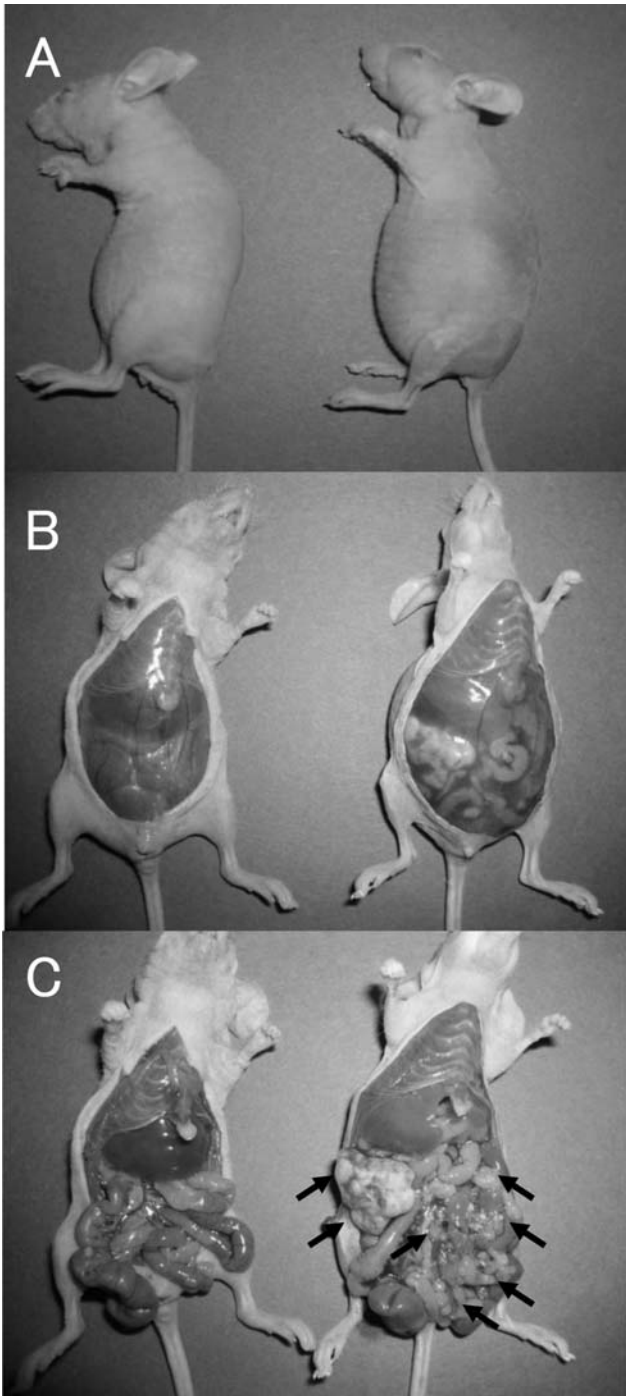


Figure 11. Peritoneal dissemination and ascites at 4 weeks after the intraperitoneal inoculation of OMC-1/IDO or control cells. The appearance of mice (A). Ascites (B). Peritoneal dissemination (C). The black arrow indicates disseminated peritoneal tumors.

administered group showed tumor enlargement, but the 1-MT-administered group did not form tumors, indicating that oral 1-MT administration inhibited OMC-1/IDO tumor growth.

Discussion

In this study, we aimed to identify a factor involved in ovarian cancer progression, particularly peritoneal dissemination, and to develop molecular therapy targeting such a factor. We

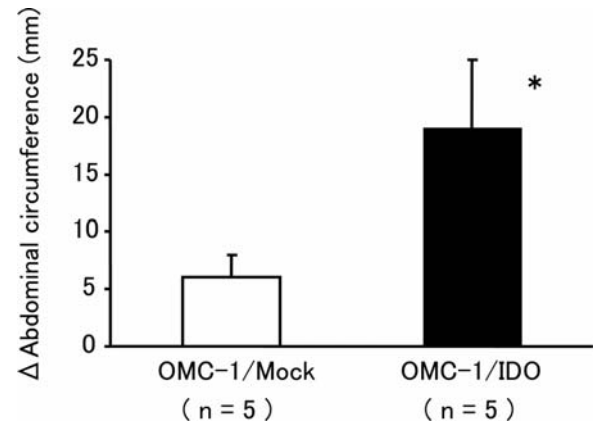


Figure 12. Changes in the abdominal circumference of mice. The increase in the abdominal circumference (19 ± 6 mm) of OMC-1/IDO cell-inoculated mice was significantly greater than that (6 ± 2 mm) of control cell-inoculated mice. * $P < 0.05$. Mean \pm SD.

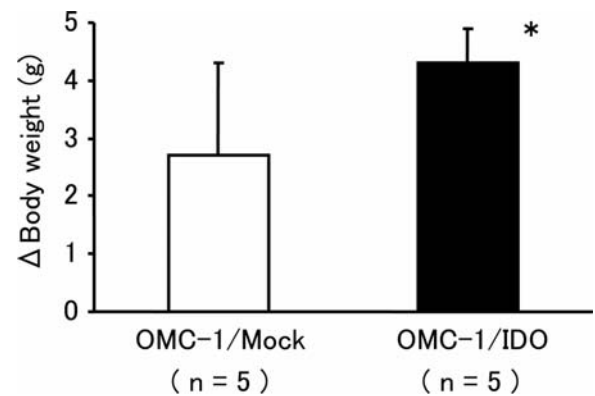


Figure 13. Changes in the body weight of mice. The increase in the body weight (4.3 ± 0.6 g) of OMC-1/IDO cell-inoculated mice was significantly greater than that (2.7 ± 1.6 g) of control cell-inoculated mice. * $P < 0.05$. Mean \pm SD.

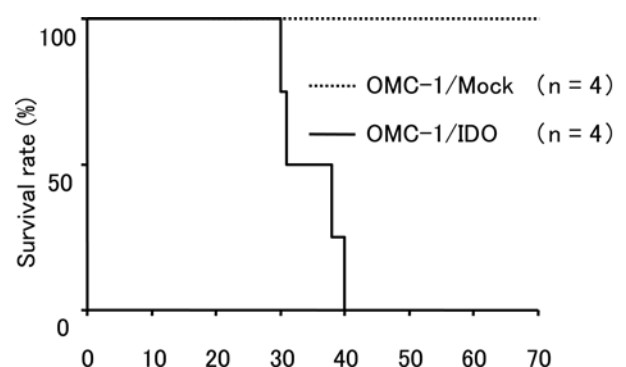


Figure 14. Survival curves of intraperitoneally inoculated mice. All control cell-inoculated mice survived for longer than 70 days, whereas all OMC-1/IDO cell-inoculated mice died of peritoneal dissemination with ascites within 40 days after inoculation.

focused on IDO, which was reported to be involved in immunotolerance, as a likely candidate, and performed a basic study of it. First, we attempted to establish an IDO-

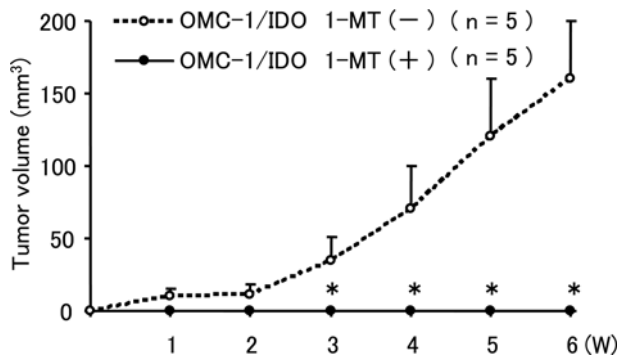


Figure 15. Subcutaneous tumor growth curve of OMC-1/IDO cells. The non-1-MT-administered group showed tumor enlargement, but the 1-MT-administered group did not form tumors. * $P < 0.05$. Mean \pm SD.

transfected ovarian cancer cell line in culture using the IDO gene to transfect the non-IDO-expressing ovarian cancer cell line OMC-1. Western blot analysis demonstrated IDO protein expression, and measurement of the tryptophan metabolite kynurenine identified the presence of IDO activity. We conducted a basic study of the IDO gene-transfected cell line thus established, and found that IDO expression did not influence ovarian cancer cell growth or invasion *in vitro*, but promoted tumor growth and peritoneal dissemination *in vivo*.

Depletion of the essential amino acid tryptophan and accumulation of its metabolite, kynurenine, inhibit cell growth and induce apoptosis. T cells are particularly sensitive to this stress, which easily suppresses their function (18). Regarding the mechanism of the immunotolerance of tumor cells, Uttenhove *et al* reported that, in IDO-expressing tumors, IDO promotes local tryptophan degradation and depletion, resulting in T-cell function suppression, leading to local immunotolerance (13). The possibility cannot be excluded that IDO expression is involved in ovarian cancer invasion, dissemination, and metastasis through such a T cell-mediated mechanism. In this basic experiment with IDO-transfected cells, we initially attempted to transplant cancer cell lines into immunocompetent mice, but no tumor formation occurred (data not shown). Therefore, we used nude mice in place of them for the experiment. Nude mice are congenitally athymic, and lack T cells; therefore, in this experimental system, we were not able to examine the effect of IDO on the T-cell lineage.

Della Chiesa *et al* reported that IDO induces the accumulation of the tryptophan metabolite kynurenine, which suppresses NK cell receptor expression, thereby inhibiting NK cell function (12). Similarly, in the present experiment, IDO expression inhibited the cytotoxic activity of NK cells *in vitro*, and suppressed NK cell accumulation in the tumor stroma *in vivo*. Thus, it appears that the IDO expression inhibited the cytotoxic activity of NK cells, thereby promoting ovarian cancer growth and peritoneal dissemination.

Angiogenesis is a very important factor for tumor growth and metastasis (19,20). An association between angiogenesis and the tumor prognosis has been reported in various malignant tumors (21), and angiogenesis-targeting molecular therapy has been clinically applied in cancers, including colorectal

cancer (22). Also, in ovarian cancer, a correlation between the intratumoral vascular density and clinical stage or prognosis was reported (23). Our previous basic studies have also shown that angiogenesis is important for ovarian cancer peritoneal dissemination, and that molecular or gene therapy targeting angiogenesis inhibits ovarian cancer peritoneal dissemination (24-27). Currently, there are very few reports on the relationship between IDO expression and angiogenesis. Only Li *et al* reported that, in the *in vitro* co-culture of vascular endothelial cells and fibroblasts with forced IDO expression, the IDO expression promoted the luminal formation of vascular endothelial cells through tryptophan depletion (28). In the present study, the number of newly formed blood vessels in tumors with IDO expression was significantly greater than that in control tumors. These findings suggest that IDO induces angiogenesis, and that IDO promotes tumor progression not only through immunotolerance but also through angiogenesis induction. Moreover, targeting IDO may lead to a novel molecular or gene therapy targeting angiogenesis inhibition.

In IDO-catalyzed tryptophan metabolism in cells, 1-MT competes with tryptophan for IDO, thereby acting as an inhibitor of IDO (29). In the present experiment, the oral administration of 1-MT to the host suppressed the tumor growth potential of IDO-transfected ovarian cancer cells with enhanced proliferative activity. Mice given 1-MT orally showed no fatal side effects. These findings suggest the possibility of IDO-targeting molecular therapy for ovarian cancer. Mueller *et al* reported that 1-MT administration to mice transplanted with IDO-overexpressing cells inhibited IDO, thereby enhancing the antitumor effect of paclitaxel (30). Since paclitaxel is currently a key drug in the chemotherapy of ovarian cancer, the combined use of such an anticancer drug and molecular-targeted therapy targeting IDO may be effective for ovarian cancer. The results of this study demonstrate that IDO is involved in ovarian cancer progression, and suggest the possibility of molecular-targeted therapy targeting IDO.

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