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ORIGINAL ARTICLE

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Evaluation of the antiangiogenic effect of Taxol in a human epithelial ovarian carcinoma cell line

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Abstract *Purpose*: Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are major ligands for the endothelium-specific tyrosine kinase receptor Tie-2 and are important regulators of endothelial cell survival. In the presence of vascular endothelial growth factor (VEGF), vessel destabilization by Ang-2 has been hypothesized to induce an angiogenic response, but in the absence of VEGF, Ang-2 leads to vessel regression. In the present study, a human ovarian cancer cell line was used to investigate the possibility that Taxol might affect the expression of Ang-1, Ang-2, and VEGF. *Materials and methods*: KF 28, a single-cell clone of a human ovarian epithelial carcinoma cell line, was used. The expression of Ang-1, Ang-2, and VEGF was assessed by quantitative real-time RT-PCR and Western blot analysis or enzyme-linked immunosorbent assay. Conditioned medium was used in the in vitro angiogenesis assay. *Results*: The concentration of Taxol that inhibited the growth of cells to the level of 50% of control cell growth was 4.65 ± 0.35 n*M*. Quantitative real-time RT-PCR indicated that *Ang-1* gene expression was significantly decreased by exposure to 2 n*M* Taxol for 168 h ($P < 0.05$ vs control cells). Western blot analysis confirmed that the Ang-1 protein level was decreased by exposure to 2 n*M* Taxol for 168 h. *Ang-2* gene expression did not significantly differ between control cells and those exposed to Taxol for any of the indicated times. The *Ang-1*/*Ang-2* gene expression ratio was significantly decreased by exposure to Taxol for 168 h ($P < 0.05$ vs control cells). *VEGF* gene expression was significantly decreased by exposure to Taxol for 168 h ($P < 0.05$). The VEGF concentration in the conditioned medium was also significantly reduced by exposure to Taxol for 168 h ($P < 0.05$). Conditioned medium collected following Taxol treatment for 168 h significantly inhibited endothelial tubule formation ($P < 0.05$). Cell growth did not significantly differ between control cells and those exposed to Taxol for any of the indicated times. *Conclusions*: Our results show that exposure of ovarian cancer cells to a low concentration of Taxol may inhibit the initiating event in angiogenesis, namely, vascular regression. This information might be valuable in the development of new therapeutic interventions for epithelial ovarian cancer.

Keywords Angiopoietin-1 · Angiopoietin-2 · Vascular endothelial growth factor · Antiangiogenesis · Taxol

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Introduction

Paclitaxel (Taxol) is a natural product derived from the bark of the Pacific yew *Taxus brevifolia* that increases the polymerization of tubulin to stabilize the cellular microtubule network. These microtubules form the mitotic spindle in cells and are therefore an important cellular element [1, 2]. Among the many drugs tested for chemotherapy of epithelial ovarian cancer in the last few years, Taxol is one of the most promising [3, 4]. Taxol has been reported to have antiangiogenic activity in xenografts at concentrations that translate to treatment doses at or below those that are administered therapeutically in patients [5, 6]. The mechanism of this antiangiogenic effect is considered to be inhibition of proliferation, motility and cord formation of endothelial cells, the angiogenic response in vivo [5, 6, 7], and neovascularization induced by vascular endothelial growth factor (VEGF) [6, 8].

The recent discovery of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) has provided novel and important insights into the molecular mechanisms of blood vessel formation [9, 10]. Ang-1 and Ang-2 share about 60% amino acid identity and bind with similar affinity to the endothelial cell tyrosine kinase receptor, Tie2 [9, 10]. Ang-1 helps to maintain and stabilize mature vessels by promoting interaction between endothelial cells and surrounding support cells, e.g. pericytes [10, 11, 12]. Ang-2 is expressed at sites of vascular remodeling and is believed to antagonize the stabilizing action of Ang-1 [10]. In the presence of VEGF, vessel destabilization by Ang-2 has been hypothesized to induce an angiogenic response. However, in the absence of VEGF, Ang-2 leads to vessel regression [10, 13, 14]. Although the exact role of the Ang/Tie2 system remains enigmatic, the available evidence indicates that, in the presence of VEGF, it is important for the initiation of angiogenesis and for vascular sprouting in tumors [10, 15].

Little is known about the effect of Taxol on this initiating event in angiogenesis. In the present study, we investigated the possibility that Taxol might affect the expression of Ang-1, Ang-2, and VEGF in a human ovarian cancer cell line.

Materials and methods

Cell culture

KF28, a single-cell clone of a human ovarian carcinoma cell line, was kindly provided by Dr. Yoshihiro Kikuchi (Department of Obstetrics and Gynecology, National Defense College). The concentration of Taxol that inhibited the growth of cells to the level of 50% of the control growth (IC_{50}) was 4.65 ± 0.35 n*M* [16]. This cell line was grown as monolayer cultures in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 m*M* glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml (Grand Island Biological Company, Grand Island, N.Y.) in a humidified atmosphere containing 5% CO_2 at 37°C, and was usually subcultured once a week with 0.25% trypsin.

Reagent preparation

Taxol was obtained from Bristol-Myers Squibb, Tokyo, Japan. It was stored at 4°C, and was added to the culture medium to give a final concentration of 2 n*M* (about 40% of the IC_{50}).

Taxol exposure

Cells ($9–10\times10^5$) were plated onto a 60×15-mm Falcon tissue culture dish (Becton Dickinson, Franklin Lakes, N.J.) 1 day before the experiments. The medium was quickly aspirated off, cells were incubated at 37°C for the indicated times without (control) or with 2 n*M* Taxol in serum-containing medium. After incubation for the indicated times, the medium was quickly aspirated off, and cell culture supernatants and cells were stored at −80°C for subsequent analysis.

RNA preparation and quantitative real-time RT-PCR procedure

Total RNA was isolated from frozen cells using a commercially available extraction kit (Isogen; Nippon Gene, Tokyo, Japan).

Complementary DNA (cDNA) was prepared by random priming from 1000 ng total RNA using a first-strand cDNA synthesis kit (Pharmacia-LKB, Uppsala, Sweden). Quantitative real-time PCR was performed using the TaqMan system (Applied Biosystems, Foster City, Calif.). The expression levels of each gene (*Ang-1*, *Ang-2*, and *VEGF*) and internal reference GAPDH were measured by multiplex PCR using TaqMan probes labeled with 6-carboxyfluorescein (FAM) or VIC, respectively. The sequences of each primer and TaqMan probe are shown in Table 1. The primers for the VEGF gene were designed from sequences within exon 2 to exon 3, which are common to all isoforms of VEGF mRNA [17]. Other primers and probes were designed using Primer Express v 2.0 software (Applied Biosystems). TaqMan Pre-Developed assay reagents, and the GAPDH primer/probe set were purchased from Applied Biosystems. Real-time PCR amplification and product detection was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems) as recommended by the manufacturer. The simultaneous measurement of each gene-FAM and GAPDH-VIC permitted normalization of the amount of cDNA added per sample. The quantity of cDNA for each experimental gene was normalized to the quantity of GAPDH cDNA in each sample. Relative expression was determined using the ΔΔCt method according to the manufacturer's protocol (user bulletin no. 2; Applied Biosystems). Each assay included a standard curve sample in duplicate, a no-template control and a cDNA sample in triplicate. All samples with a coefficient of variance higher than 10% were retested.

Western blot analysis

The expression of Ang-1 and Ang-2 proteins in total cell lysates was determined by Western blotting. Cultured cells were washed in serum-free phosphate-buffered saline (PBS) and dissolved in lysis buffer (50 m*M* Tris-HCl, pH 7.4, 125 m*M* NaCl, 0.1% NP-40, 5 m*M* NaF, 1 m*M* PMSF, 1 ng/ml leupeptin, 1 ng/ml aprotinin) for 60 min on ice. The lysates were centrifuged at 2500 *g* for 5 min. Homogenates of human placental tissues were prepared by the same procedure and were used as a positive control for Ang-1 and Ang-2. Protein concentrations were measured using a Bradford protein assay kit (BioRad Laboratories, Hercules, Calif.) with bovine serum albumin as the standard protein. Ang-1 or Ang-2 protein (100 μg) or *β*-actin protein (50 μg) were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride filter (Millipore, Bedford, Mass.). After blocking with 5% skimmed milk in PBS including 0.1% Tween 20 for 2 h at room temperature, the blotted membranes were incubated with specific antibodies. The antibodies used in this study were anti-Ang-1 rabbit polyclonal

Table 1 Oligonucleotide primers and TaqMan probes used to quantitate cDNA

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	TaqMan probe (5′-3′)
Ang-1	GTTAACAGGAGGATGGTGGTTTG	TGGTTTTGTCCCGCAGTATAGAA	TGCTTGTGGCCCCTCCAATCTAAATG
Ang-2	ATGGGTCCTGCAGCTACACTTT	TGCACAGCATTGGACACGTA	ACAACTGCCGCTCTTCCTCCAGCC
VEGF	GCACCCATGGCAGAAGG	CTCGATTGGATGGCAGTAGCT	TTCATGGATGTCTATCAGC

antibody, anti-Ang-2 rabbit polyclonal antibody, both of which were kindly provided by Dr. George D. Yancopoulos (Regeneron Pharmaceuticals, Tarrytown, N.Y.), and anti-β-actin mouse monoclonal antibody (AC-15, 1:2000 dilution) purchased from Sigma-Aldrich (St. Louis, Mo.). Blots were developed with peroxidase-labeled anti-rabbit or anti-mouse IgG antibody (1:1000) purchased from MBL (Nagoya, Japan). After extensive washing, bands were detected using an enhanced chemiluminescence system (ECL detection system; Amersham Biosciences, Little Chalfont, UK).

Measurement of VEGF in cell culture supernatants

Levels of VEGF were assayed in cell culture supernatants by a solid phase quantitative sandwich enzyme immunoassay technique (Quantikine Human VEGF Immunoassay; R & D Systems, Minneapolis, Minn.). According to the manufacturer's explanatory note, this kit can be used to determine relative mass values for natural human VEGF. Briefly, 200 µl diluted recombinant human VEGF or cell culture supernatant was pipetted into a microtiter plate coated with monoclonal antibody specific for human VEGF and incubated for 2 h at room temperature. After three washes, 200 µl of an enzyme-linked polyclonal antibody specific for VEGF was added to each of the wells to sandwich the bound VEGF. After three further washings to remove any unbound antibody-enzyme reagent, tetramethylbenzidine was added as a substrate. After 20 min incubation at room temperature, the color development reaction was stopped and the intensity of the color was measured by reading the absorbance at 450 nm (Molecular Devices Corporation, Sunnyvale, Calif.). Each measurement was done in duplicate, and the VEGF level was calculated from a standard curve generated using the program SOFTmax PRO (version 2.4) on a Macintosh computer.

Measurement of in vitro angiogenesis

A 24-multiwell tissue culture plate format originally designed for the assessment of in vitro angiogenesis (KZ-1000; KURABO, Osaka, Japan) was used for this study. In this assay, human umbilical endothelial cells (HUVECs) and human diploid fibroblasts of dermal origin were cocultured. The system does not require the addition of further growth factors other than those normally present in endothelial growth medium or any matrix protein. After examining the cultures microscopically for cell morphology and signs of growth of the endothelial cells, diluted (1:1) conditioned medium was added to the treatment wells. Two wells were assigned for each of the control (endothelial cell medium only), positive control (rVEGF-A 10 nM/ml) and negative control (rVEGF-A 10 nM/ml + Suramin 50 μM/ml) groups. The medium was changed every 3 days using diluted conditioned medium. After 11 days, cells were fixed with ice-cold 70% ethanol and endothelial cell tubules were stained with a staining kit (ZHA-1225; KURABO) according the manufacturer's protocol. A primary antibody against human CD31 at a dilution of 1:4000 was used for detection of the endothelial cells. Visualization of the staining was done with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) solution. Digital images were captured from four quadrants of each well using a charge-coupled device video camera (HC-300/OL; Olympus, Tokyo, Japan) and color image freezer computer software (Photograb-300 SH-3; Fujifilm, Tokyo, Japan), and stored on optical disks. Quantification of the CD31-stained area was performed using a public domain National Institutes of Health (NIH) image program (developed at the US NIH and available from URL: http://rsb.info.nih.gov/nih-image/) on a MacIntosh computer. The average of four measurements for each well was calculated and the results are expressed as the number of pixels per unit area of field.

MTT assay

Cells were seeded at 3×10^3 in a volume of 180 µl of serum-containing medium per well into 96-well microplates (Corning, New York, N.Y.) and incubated at 37°C for 24 h to adhere. The medium was then replaced by 180 µl serum-containing medium without (control) or with 2 nM Taxol, and incubated at 37°C for the further indicated times.

After incubation for the times indicated, 20 µl MTT (5 mg/ml) (Sigma, St. Louis, Mo.) was added to each well, the plates were incubated at 37°C for 4 h and the medium was aspirated off. The resulting formazan crystals were dissolved using 200 µl MTT solubilization solution (Sigma), and the plates were agitated for 5 min. The absorbance was then read on a scanning spectrophotometer (SOFTmax Pro; Molecular Devices, Sunnyvale, Calif.) using a test wavelength of 550 nm and a reference wavelength of 620 nm.

Statistical analysis

Each experiment included at least three independent determinations for cells of separate dishes or microplates. The same experiments were repeated a minimum of two or three times. Similar results were obtained from experiment to experiment. The value for the effect of each compound tested is expressed as a percentage of the mean of the control replicates for that experiment, and the data are shown as means ± SE. The Mann-Whitney U-test was used as appropriate for the evaluation of differences between end-points. P values less than 0.05 were considered significant.

Results

Ang-1 and *Ang-2* expression in KF28 cells after Taxol exposure

Ang-1 and *Ang-2* gene expression as determined by quantitative real-time RT-PCR in cells with or without exposure to Taxol was investigated at 24, 48, 72, 96, 120, 144, and 168 h. *Ang-1* gene expression was significantly decreased by exposure to Taxol for 168 h (percent of control: mean 55.5, SE 1.95, $P<0.05$; Fig. 1a). Next, Western blot analysis confirmed that the level of Ang-1 protein was decreased following exposure to Taxol for 168 h (Fig. 1b). The Western blot results coincided well with those obtained by quantitative real-time RT-PCR. *Ang-2* gene expression in cells exposed to Taxol for any of the indicated times did not significantly differ the expression in control cells. Figure 2 shows the results for Ang-2 of quantitative real-time RT-PCR and Western blot analysis, respectively, following exposure to Taxol for 168 h. The *Ang-1*/*Ang-2* gene expression ratio, calculated separately for matched experiments, was significantly decreased by exposure to Taxol for 168 h (percent of control: mean 56.5, SE 7.36, $P<0.05$; Fig. 3).

VEGF expression and production in KF28 cells after Taxol exposure

VEGF gene expression was significantly decreased by exposure to Taxol for 168 h (percent of control: mean 34.7, SE 0.55, $P<0.05$; Fig. 4a). VEGF concentration in

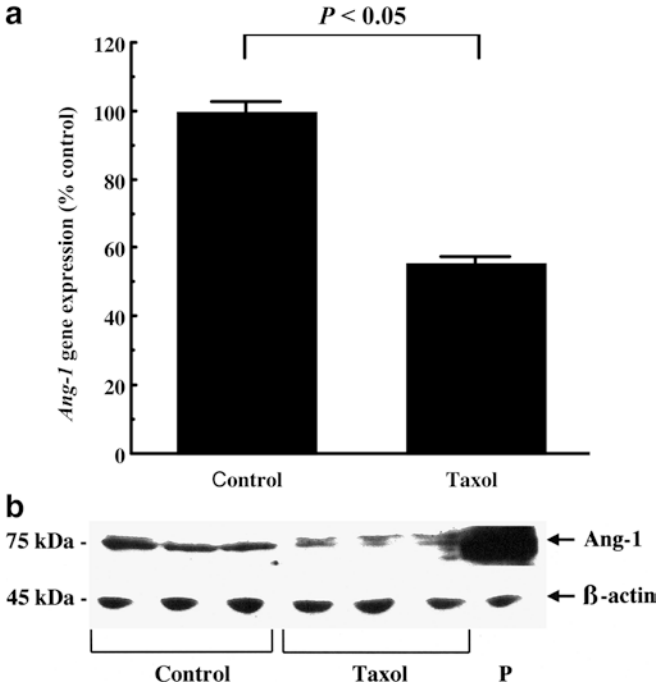


Fig. 1 **a** *Ang-1* gene expression by quantitative real-time RT-PCR in cells exposed to Taxol for 168 h and in cells (control) not exposed. The results are presented as means + SE ($n = 3$). **b** Western blot analysis of the expression of Ang-1 in cells exposed to Taxol for 168 h and in cells (control) not exposed (*P* human placenta at 38 weeks gestation)

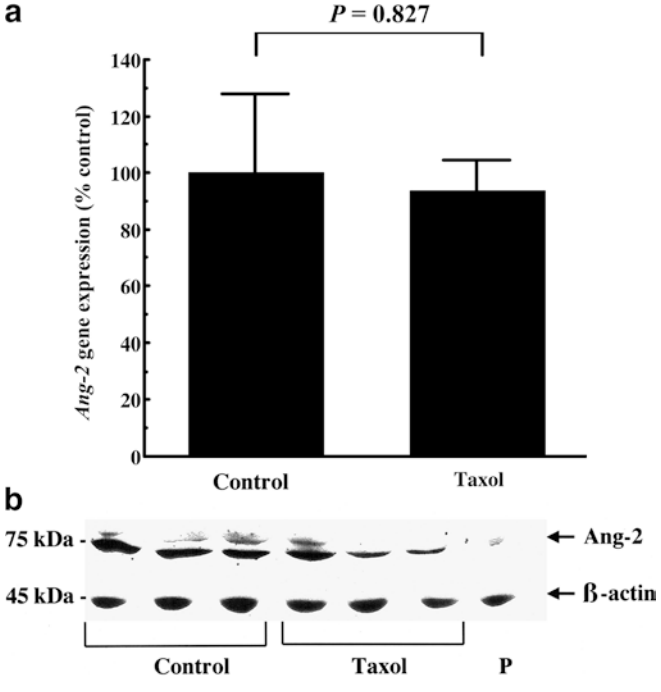


Fig. 2 **a** *Ang-2* gene expression by quantitative real-time RT-PCR in cells exposed to Taxol for 168 h and in cells (control) not exposed. The results are presented as means + SE ($n = 3$). **b** Western blot analysis of the expression of Ang-2 in cells exposed to Taxol for 168 h and in cells (control) not exposed (*P* human placenta at 38 weeks gestation)

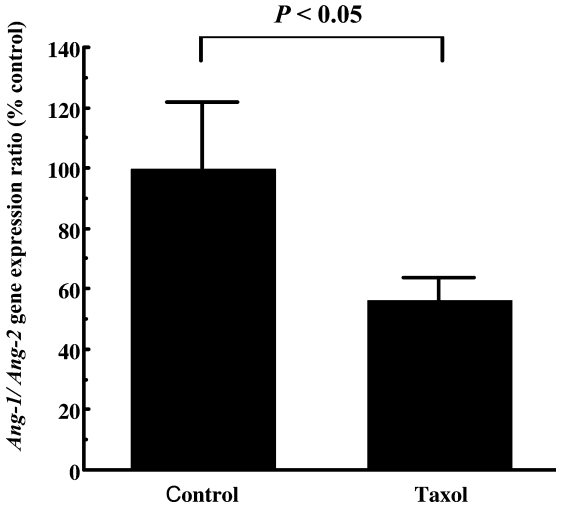


Fig. 3 *Ang-1*/*Ang-2* gene expression ratio by quantitative real-time RT-PCR in cells exposed to Taxol for 168 h and in cells (control) not exposed. The results are presented as means + SE ($n = 3$)

the conditioned medium was also significantly reduced by exposure to Taxol for 168 h (percent of control: mean 42.5, SE 8.76, $P < 0.05$; Fig. 4b).

In vitro angiogenesis in KF28 cell line after Taxol exposure

To examine the functional relevance in KF28 cells of Taxol exposure, we examined the antiangiogenic activities of the conditioned medium in an in vitro angiogenesis model. Vascular tubule formation following VEGF treatment was efficiently inhibited by suramin treatment (percent of VEGF treatment: mean 42.3, SE 3.83, $P < 0.05$). As shown in Fig. 5, conditioned medium collected following Taxol treatment for 168 h significantly inhibited endothelial tubule formation (percent of control: mean 50.1, SE 1.94, $P < 0.05$).

Growth of KF28 cells after Taxol exposure

Cell growth did not significantly differ between control cells and those exposed to Taxol for any of the indicated times (data not shown).

Discussion

It has been proposed that the Ang/Tie2 system might regulate the interaction of endothelial cells with surrounding mesenchymal cells, e.g. pericytes [13]. Pericytes restrict endothelial cell proliferation by forming a physical barrier, and they have been shown to inhibit endothelial cell migration [18]. Initiation of vessel sprouting in adults is preceded by a local drop-off of pericytes from the existing vessel that enables endothe-

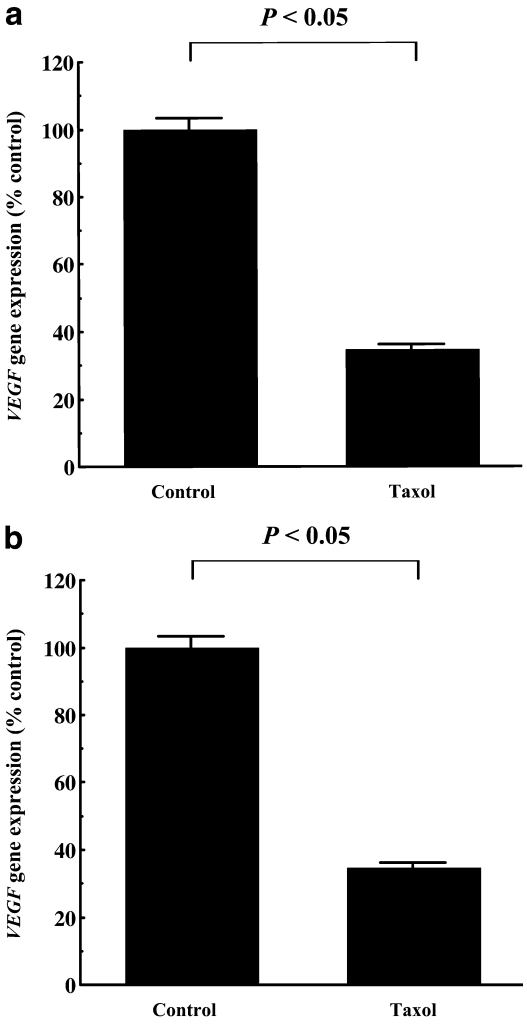
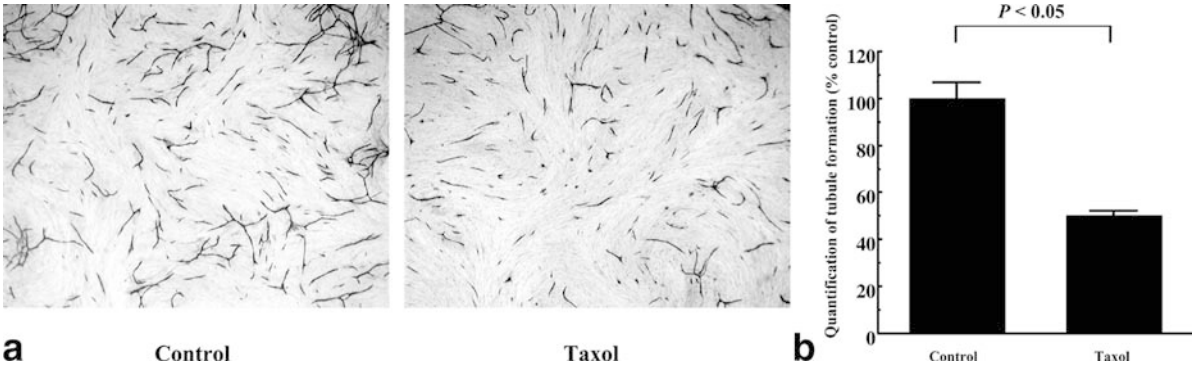


Fig. 4 **a** *VEGF* gene expression by quantitative real-time RT-PCR in cells exposed to Taxol for 168 h and in cells (control) not exposed. The results are presented as means + SE ($n = 3$). **b** VEGF concentration in conditioned medium following exposure to Taxol for 168 h and that in conditioned medium of control cells. The results are presented as means + SE ($n = 3$)

Fig. 5 **a** Representative photographs of tubule formation in conditioned medium following exposure to Taxol exposure for 168 h and in conditioned medium of control cells (×20). **b** Quantification of tubule formation in conditioned medium following exposure to Taxol exposure for 168 h and in conditioned medium of control cells. The results are presented as means + SE ($n = 3$)

lial cells to overcome growth inhibition and start proliferating and migrating [19, 20]. It has been suggested that a physiological role of Ang-1 could be to recruit pericytes and support the physical association of endothelial cells and pericytes [10]. Inhibition of Ang-1 by Ang-2 drives pathological angiogenesis in the presence of angiogenesis inducers such as VEGF by loosening contacts between endothelial and periendothelial cells [10, 13, 14]. In a previous study, we have found that *Ang-1* gene expression and the *Ang-1*/*Ang-2* gene expression ratio, assessed by conventional RT-PCR, are both significantly higher in normal ovary with corpus luteum (CL) than in ovarian cancer, whereas the expression levels of the *Ang-2* and *Tie2* genes are not significantly different between normal ovary with CL and ovarian cancer [21]. We speculated that the decreased expression of Ang-1 in ovarian cancer might prompt pericyte drop-off by Ang-2, which is thought to be a requirement for rendering and maintaining endothelial cells accessible to pathological angiogenic inducers [21].

In this study, *Ang-1* gene expression, as assessed by quantitative real-time RT-PCR, was found to be significantly decreased following exposure to Taxol for 168 h compared to the control. Subsequently, Western blot analysis confirmed that the level of Ang-1 protein was decreased by exposure to Taxol for 168 h. Ang-1 mRNA expression correlated with Ang-1 protein expression. This correlation was also noted with Ang-2 and VEGF. *Ang-2* gene expression did not significantly differ between control cells and those exposed to Taxol for the indicated times. Moreover, the *Ang-1*/*Ang-2* gene expression ratio was significantly decreased compared to the control following exposure to Taxol for 168 h. As Ang-1 acts via the Tie2 receptor to recruit pericytes and support the physical association of endothelial cells with pericytes [10], this overexpression of Ang-2 is thought to block the constitutive stabilizing action of Ang-1 on vessels. It has been proposed that destabilization by Ang-2 in the absence of VEGF leads to frank vessel regression, whereas such destabilization in the presence of high VEGF levels facilitates the angiogenic response [10, 13, 14]. Following exposure to Taxol for 168 h, *VEGF* gene expression and VEGF concentration in the conditioned medium were significantly reduced. Moreover, conditioned medium from cells following exposure



to Taxol for 168 h significantly inhibited endothelial tubule formation.

The angiopoietins, Ang-1 and Ang-2, and VEGF are known growth factor families that are specific for the vascular endothelium because expression of their receptors is restricted to these cells [10, 21, 22]. The results obtained from the in vitro investigations showed a relative upregulation of Ang-2 compared to Ang-1 and downregulation of VEGF. The changes in expression of these ligands after Taxol exposure might induce inhibition of angiogenesis via their receptors in the in vitro assay. However, some issues are still unresolved. Why was no effect observed at shorter times? Keyes et al. [23] examined VEGF secretion after exposure to Taxol for 72 h at several concentrations in exponential and stationary phase human CaKi-1 renal cell carcinoma cells and human SW2 small-cell lung carcinoma cells. VEGF secretion by only exponential SW2 cells decreased in a concentration-dependent manner and VEGF levels did not decrease with treatment in the other cells. Although we used only one cell line and one concentration (2 n*M*) in this study, with the characteristics of KF28 cells and under the experimental conditions used in this study, modulation of gene expression and protein production with Ang-1 and VEGF might be expected after exposure to Taxol for 168 h. Was the reduction of Ang-1 and VEGF expression sufficient to cause a biological effect? It is possible that the antiangiogenic effect attributed to the conditioned medium might have been due to the direct effect of Taxol remaining in the medium rather than due to modulation of the angiogenic factors by Taxol. However, it was shown that the dose of Taxol used (2 n*M*) had no antiproliferative effect on KF28 cells, and in the antiangiogenic assay the conditioned medium was further diluted with endothelial growth medium. Therefore, the antiangiogenic effect of Taxol could be attributable to downregulation of angiogenic growth factors in the conditioned medium.

In the experiments reported here we showed the possibility that exposure of ovarian cancer cells to a low concentration of Taxol (2 n*M*, about 40% of IC_{50}) inhibited the initiating event in angiogenesis, namely, vascular regression. Clear cytotoxicity was absent because cell growth did not significantly differ between control cells and those exposed to Taxol for any of the indicated times. This means that Taxol might conceivably affect vascular regression at lower concentrations than those necessary to cause a cytotoxic effect in tumor cells. The use of low doses in 'metronomic' chemotherapy (namely, very frequent or continuous low-dose chemotherapy) as an antiangiogenic targeting strategy seems particularly effective against drug-resistant tumors. In addition, low-dose chemotherapeutic agents for metronomic therapy would not be expected to be associated with acute toxicity and side effects [24, 25, 26]. More studies are needed to clarify the therapeutic relevance of our findings and to develop treatment strategies.

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