A Novel Snake Venom Disintegrin That Inhibits Human Ovarian Cancer Dissemination and Angiogenesis in an Orthotopic Nude Mouse Model

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Key Words

Ovarian cancer · Adhesion · Invasion · Integrin · Snake venom · Disintegrin · Angiogenesis · Animal model

Abstract

OVCAR-5 is a human epithelial carcinoma cell line of the ovary, established from the ascitic fluid of a patient with progressive ovarian adenocarcinoma without prior cytotoxic treatment. The unique growth pattern of ovarian carcinoma makes it an ideal model for examining the anticancer activity of conortrostatin (CN), a homodimeric disintegrin from southern copperhead venom. FACS analysis revealed that OVCAR-5 is integrin $\alpha v \beta 3$ negative, but $\alpha v \beta 5$ positive. CN effectively blocks the adhesion of OVCAR-5 cells to several extracellular matrix proteins and inhibits tumor cell invasion through an artifi-

cial basement membrane. In a xenograft nude mouse model with intraperitoneal introduction of OVCAR-5 cells, intraperitoneal injection of CN was used for therapy. Tumor dissemination in CN-treated versus control groups was studied by gross examination, and antiangiogenic potential was examined by factor VIII immunohistochemistry and image analysis. CN not only significantly inhibited ovarian cancer dissemination in the nude mouse model, but it also dramatically prevented the recruitment of blood vessels to tumors at secondary sites.

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Introduction

Disintegrins are small, disulfide-rich, Arg-Gly-Asp (RGD)-containing peptides from snake venom [1] that bind to cell adhesion

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receptors (integrins) on the surface of normal and malignant cells [2–4]. Disintegrins have been characterized from the venom of many snakes and were originally distinguished by their ability to inhibit platelet aggregation [3, 5]. We have purified and characterized the disintegrin, contortrostatin (CN), from southern copperhead snake venom [6]. CN gives a single band of approximately 14–15 kD by nonreducing SDS-PAGE and approximately 6–7 kD by reducing SDS-PAGE. Mass spectrometry indicated a mass of 13,505 for intact CN and 6,956 for the reduced and pyridylethylated protein, indicating that it is a homodimer, a structure unique among all other disintegrins reported to date. We have determined the primary structure of CN [7]. In addition to α Ib β 3 on blood platelets, integrins α 5 β 1, α v β 3 and α v β 5 on tumor cells have been shown to be binding sites for CN [8–10].

Integrins are important cell surface receptors that are involved in cell-cell and cell-matrix interactions [11]. All integrins are α / β heterodimeric glycoproteins [12]; different α -subunits combine with several distinct β -subunits resulting in a range of specificity toward distinct extracellular matrix (ECM) proteins [11]. Several classes of integrins recognize the tripeptide RGD sequence present in ECM proteins [13]. Although originally believed to be inert molecules, integrins are known to link the ECM with cytoskeletal proteins and to be involved in bidirectional signaling that alters cellular functions [14].

In previous investigations, we reported that CN binds to integrins and blocks the adhesion of human breast cancer cells (MDA-MB-435) to the ECM [9]. CN also prevents invasion of MDA-MB-435 cells through an artificial basement membrane. Daily local injection of CN into MDA-MB-435 tumor masses in an orthotopic xenograft nude mouse model significantly inhibits tumor

growth and reduces the occurrence of pulmonary metastasis. We have identified α v β 3, an important integrin mediating cell motility and tumor invasion, as one of the binding sites for CN on MDA-MB-435 cells. We hypothesized that CN inhibits the progression of breast cancer, at least in part, by an antiangiogenic action, and we have shown in the xenograft nude mouse model that CN dramatically inhibits breast cancer-induced angiogenesis [9]. In the present study, we report that CN has effective antitumor activity in vitro and in vivo in a human ovarian cancer cell line, OVCAR-5. Studies about OVCAR-5 utilized a xenograft model established by Hamilton et al. [15]. Immunohistochemistry was used to demonstrate that CN significantly inhibited angiogenesis in the ovarian cancer animal model.

Materials and Methods

Materials

Venom from *Agkistrodon contortrix contortrix* was purchased from Miami Serpentarium Laboratories (Punta Gorda, Fla., USA). Contortrostatin was purified by four-step high-performance liquid chromatography [6, 8]. Fibronectin (FN), vitronectin (VN) and MatrigelTM were from Becton Dickinson (Bedford, Mass., USA). Monoclonal antibodies (mAbs) PID6 (anti- α 5) and P4C10 (anti- β 1) were purchased from Gibco Life Technologies (Gaithersburg, Md., USA), and P1F6 (anti- α v β 5) was from Chemicon International (Temecula, Calif., USA). MAb 7E3 (an anti- α Ib β 3 antibody that has a high degree of cross-reactivity to α v β 3) was kindly provided by Dr. Marian Nakada (Centocor, Malvern, Pa., USA). Goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) was purchased from Jackson ImmunoResearch (West Grove, Pa., USA). The monoclonal rabbit anti-human antibody against factor-VIII-related antigen, goat anti-rabbit secondary antibody, and immunohistochemistry detection kit (HistoMouse-SP kit) were purchased from Zymed Laboratories, Inc. (Gilroy, Calif., USA).

Cell Culture

OVCAR-5 human ovarian carcinoma cell line was generously provided by Dr. Tom Hamilton (Fox Chase Cancer Center, Philadelphia, Pa., USA). OVCAR-5 cells were derived from a patient with advanced epithelial ovarian cancer with no prior cytotoxic therapy [15]. The cells were grown in RPMI with 10% fetal bovine serum and Premix.

Characterization of Integrin Profile by Flow Cytometry

Standard FACS analysis with specific anti-integrin antibodies was employed to detect integrins displayed by OVCAR-5 cells.

Adhesion of OVCAR-5 Cells to CN and ECM Protein

The level of cell adhesion supported by different CN concentrations (0.1, 0.5, 1, 5 $\mu\text{g/ml}$) was determined experimentally by immobilizing CN onto Immulon-II 96-well microtiter plates (Dynex Technologies, Inc., Chantilly, Va., USA). Excess protein was washed off and unbound sites were blocked with 1% bovine serum albumin. OVCAR-5 cells ($100\ \mu\text{l}$, 10^5 cells/ml) were seeded in the coated wells (1 h, 37°C). Unbound cells were washed off with three washings of PBS. Cell adhesion was determined by Cell Titer 96TM AQueous Nonradioactive Cell Proliferation Assay kit (Promega, Madison, Wisc., USA).

VN (15 $\mu\text{g/ml}$) and FN (15 $\mu\text{g/ml}$) were immobilized onto Immulon-II 96-well microtiter plates. Unbound sites were blocked with 1% bovine serum albumin in PBS. OVCAR-5 cells in serum-free medium (SFM) were treated with different concentrations of CN (0, 1, 10, 100, 1,000 nM) for 30 min at room temperature, added to each well of microtiter plates and treated as described above. All tests were repeated at least three times in triplicate.

Invasion Assay

Transwells (Costar, Cambridge, Mass., USA) were coated with Matrigel (1:50) in SFM and dried in the hood overnight. Membranes were rehydrated with $500\ \mu\text{l}$ of SFM 1 h before cells were added. Cells were trypsinized, washed in serum-containing medium, and resuspended in SFM. Different concentrations of CN (0, 10, 100, 1000 nM) were used to treat OVCAR-5 cells (30 min at room temperature). MAbs, anti- $\alpha\text{v}\beta 3$ (7E3) and anti- $\alpha\text{v}\beta 5$ (PIF6), were used separately to treat cells for 30 min before cell suspensions were added to the top chamber; HT1080-conditioned medium (600 μl) was in the bottom chamber. Untreated OVCAR-5 cells, with HT1080-conditioned medium in

the bottom chamber, was used as a positive control, and untreated OVCAR-5 cells, with SFM in the bottom chamber, was used as a negative control. Plates were incubated for 8 h to determine the level of cell migration. After incubation, inserts were removed and stained with 0.2% crystal violet aqueous solution in 20% methanol for 30 min. After washing, noninvaded cells from the upper surface of the membrane were removed with cotton swabs. Invaded stained cells were dissolved in 250 μl of 1% SDS and 200 μl were transferred to a 96-well plate. Optical density at 570 nm was measured in a microplate reader.

Animal Model

Five-week-old female nude mice (BALB/c/nu/nu) were purchased from Simonsen Lab (Gilroy, Calif., US). For the humane treatment of experimental animals, a protocol approved by the Institutional Animal Care and Use Committee, University of Southern California, USA, was followed. Animals were kept in a pathogen-free environment, and fed sterilized food and water. OVCAR-5 cells were washed and resuspended in sterile PBS. Cell suspension (10^6 cells/mouse, 0.2 ml/mouse) was injected into the peritoneal cavity.

The animal study used 22 five-week-old female nude mice. Both the control and treated groups contained 11 mice. Implanted tumors were allowed to grow for 1 week prior to the initiation of treatment. The treated group received twice daily injections of 200 μl of CN (0.1 mg/ml, 40 $\mu\text{g/day}$). The control group received twice daily injections of physiological saline (200 $\mu\text{l/injection}$). Treatment was continued for a total of 4 weeks, at which time the nude mice were anesthetized by inhalation of Metofane (Pitman-Moore, Mundelein, Ill., USA) in a closed chamber and euthanized for angiogenic study. Photographs were taken to document tumor load. Tumor tissue from the peritoneal cavity was collected, weighed, and stored in 4% formalin at 4°C for factor VIII immunohistochemistry.

Microvessel Density Analysis

Tumor masses from the in vivo study were fixed in phosphate-buffered 10% formalin, embedded in paraffin, sectioned at 4 μm , and processed for immunohistochemistry. Slides were prepared for staining by treatment with xylene and a graded alcohol series. Specimens were placed in 3% H_2O_2 to quench endogenous peroxidase activity and treated with citrate buffer. Slides were incubated with 100 μl of rabbit anti-factor VIII mAb overnight (37°C), treated with secondary goat anti-rabbit antibody, and developed by the standard peroxidase procedure.

Fig. 1. Inhibition of adhesion of OVCAR-5 cells to immobilized VN and FN. Pretreatment of OVCAR-5 cells with CN for 30 min (0–1,000 nM CN) inhibited adhesion of OVCAR-5 cells (100 μ l of cells, 10^5 cells/ml) to immobilized ECM proteins following 1 h of incubation. The IC_{50} for CN inhibition of OVCAR-5 adhesion to immobilized VN was 1 nM, and to immobilized FN 10 nM. CN at similar concentrations did not inhibit adhesion to immobilized laminin or collagen type I.

Image Analysis

'Hot spots' were selected within each sample slide at 100-fold magnification with a Zeiss light microscope and area measurements (pixels) were made using a Leica Q570 image analyzer. 'Hot spots' are defined as areas of high vessel density [9, 16]. To reduce bias in this study, analysis of the slides was done in a blind fashion.

Results

OVCAR-5 Integrin Profile

Primary mAbs against integrins $\alpha v \beta 3$ (7E3), $\alpha v \beta 5$ (P1F6), $\alpha 5$ (P1D6), and $\beta 1$ (P4C10) were incubated with OVCAR-5 cells; bound antibodies were detected by secondary FITC-labeled antibody. Flow cytometry results showed that OVCAR-5 cells do not express integrin $\alpha v \beta 3$. This is an interesting

result because previous studies have pointed to the importance of this integrin for tumor cell migration [17, 18]. However, OVCAR-5 cells do express $\alpha v \beta 5$, $\alpha 5$ and $\beta 1$.

Contortrostatin Inhibits OVCAR-5

Adhesion to Immobilized ECM Proteins

OVCAR-5 cells were pretreated with CN at different concentrations for 30 min before incubation with immobilized VN or FN. Although OVCAR-5 cells adhered to VN and FN, CN inhibited adhesion to both ECM proteins dose dependently: FN IC_{50} = 10 nM, VN IC_{50} = 1 nM (fig. 1). Although OVCAR-5 adhered to laminin and collagen I, CN was unable to inhibit this adhesion.

Contortrostatin Inhibits OVCAR-5

Invasion

In order to find the optimal invasion time for OVCAR-5 cells across MatrigelTM-coated Transwells, several incubation times were tested and a time of 8 h was chosen for analysis. Under these conditions, CN (1,000 nM) pretreatment of the OVCAR-5 cells was found to inhibit cell migration by about 60% (fig. 2). 7E3 (mAb to $\alpha v \beta 3$) did not inhibit cell migration, but P1F6 (mAb to $\alpha v \beta 5$) at 100 nM inhibited migration to approximately the same extent as 1,000 nM CN.

Animal Model Study

In order to prevent the occurrence of necrosis that appears in the later stages of ovarian tumor growth, a 4-week treatment schedule was planned, beginning 1 week after the initial tumor injection. In the treatment group (11 animals), CN was administered at the level of 20 μ g twice per day. Control animals received intraperitoneal saline injections similarly administered. At the conclusion of the study after 4 weeks of treatment, 16 mice (8 in each group) were sacrificed. For various reasons, 3 mice in each group were excluded

Fig. 2. Inhibition of OVCAR-5 cell invasion through an artificial basement membrane (Matrigel) using a Boyden chamber. Thirty-minute pretreatment of OVCAR-5 cells with varying concentrations of CN (0, 10, 100 and 1,000 nM) inhibited migration across a Matrigel barrier, measured after 8 h. HT1080-conditioned medium served as a chemoattractant in the bottom chamber. Untreated OVCAR-5 cells with HT1080-conditioned medium in the bottom chamber

was used as a positive control, and untreated OVCAR-5 cells with SFM in the lower chamber was used as a negative control. Pretreatment of OVCAR-5 cells for 30 min with anti- $\alpha\text{v}\beta 3$ mAb (7E3) did not inhibit OVCAR-5 invasion significantly, whereas an anti- $\alpha\text{v}\beta 5$ mAb (P1F6) at 100 nM inhibited cell invasion by about 60%, approximately the same as that achieved by 1,000 nM CN.

from the study. A representative mouse was chosen from each group for photography (fig. 3). It is obvious that CN significantly decreased tumor burden in the CN-treated group. Tissue from 4 representative mice from the remaining mice in each group (control and CN-treated) were formalin fixed for histological examination. Histological sections were stained with antibody against factor-VIII-related antigen as a specific marker of vascular endothelial cells. One of the most remarkable differences between CN-treated and control groups was the complete absence of tumor in various organs of some of the CN-treated animals. Thus, these organs in the animals did not have 'hot spots' of tumor in which microvessel growth could be detected.

The paucity of tumor in some organ specimens resulted in a zero microvessel count. Moreover, in the CN-treated specimens in which tumor did grow, the microvessel density was dramatically lower than that found in the untreated group (table 1).

Discussion

Ovarian cancer is usually asymptomatic and not diagnosed until the cancer has spread outside of the ovary. About 70% of patients with epithelial ovarian cancer present as stage III or IV (advanced disease). Because of this, ovarian cancer has been called a silent killer; it is the leading cause of death from gynec-

Fig. 3. In vivo model of OVCAR-5 in nude mice. Treated mice (**A**) showed significantly fewer nodules than control mice (**B**). OVCAR-5 (1×10^6 cells/mouse) implanted intraperitoneally. Treatment consisted of 20 μ g of CN per mouse twice daily for 4 weeks starting 1 week after tumor injection. Arrows point to tumor nodules.

Table 1. Total factor VIII staining area, by treatment and tumor location, determined by image analysis and area measurement (pixels)

Treatment	Intestine	Ovary	Spleen	Stomach	Total
CN			728.9		728.9
CN			3,128.3		3,128.3
CN			1,771.7	1,042.8	2,814.5
CN		1,779.3			1,779.3
Control (saline)	49,439.5		15,580.8		65,016.3
Control (saline)	2,260.2	25,841.5			28,101.7
Control (saline)				10,369.5	10,369.5
Control (saline)	14,540.6	587.2	25,094.9		40,222.6

A blank indicates that no tumor developed at this site.

logical malignancy resulting in 14,800 deaths per year in the United States [19]. It is apparent that present methods of treatment for advanced-stage ovarian cancer are not effective, and novel therapeutic approaches to control growth and spread are needed for this most devastating cancer in women.

The role of angiogenesis in solid tumor growth and dissemination has long been known [20, 21], and recently the understanding that tumor growth is angiogenesis-dependent [20] has become a dogma. Folkman [22] proposed a two-compartment tumor model composed of vascular endothelial cell and tumor cell compartments. Angiogenesis appears to be an early event in epithelial ovarian cancer [23] and plays an important role in ovarian cancer growth and dissemination [24]. Angiogenesis has been correlated with prognosis in ovarian cancer patients, a higher vessel count shows a trend to worse overall survival in advanced ovarian cancer [23, 25–27]. Antiangiogenic therapy represents a potentially effective therapy for long-term control of ovarian cancer [28].

The importance of VN receptors in angiogenesis is well known. It has been shown that an mAb to integrin $\alpha\beta 3$, as well as a cyclic RGD-containing peptide, perturbed angiogenesis and produced regression of a human cancer growing on the chick embryo chorioallantoic membrane [29]. In previous studies, we have shown that CN effectively blocked adhesion of human umbilical vein endothelial cells to immobilized VN and significantly inhibited invasion of human umbilical vein endothelial cells through a Matrigel barrier [30]. Competitive binding assays and adhesion assays with different integrin antibodies suggested that integrin $\alpha\beta 3$ is a binding site for CN on vascular endothelial cells. We conclude that CN binds to $\alpha\beta 3$, and interferes with the anchorage-dependent survival of the vascular endothelial cells and the mobility of

the cells. The consequent suppression of angiogenesis is an important component of the antineoplastic activity of CN [30].

It has also been suggested that integrins play an important role in intraperitoneal dissemination of ovarian cancer [31], as demonstrated by the finding that multiple intraperitoneal injections of an RGD peptide inhibited experimental peritoneal seeding of human ovarian cancer cells *in vivo* [32]. *In vitro* studies suggested that $\beta 1$ and $\beta 3$ integrins are important in the interaction of ovarian cancer cells with metastatic sites on the mesothelium [33]. We have shown that OVCAR-5 is $\alpha\beta 3$ negative; however, CN is able to block invasion of these cells due to its ability to inhibit the function of $\alpha\beta 5$ [10]. The role of this integrin ($\alpha\beta 5$) in tumor invasion has recently been reported [34]. OVCAR-5 is $\alpha\beta 5$ positive, and it is apparently through this integrin that CN exerts its inhibitory activity on OVCAR-5 adhesion and invasion.

Human ovarian carcinoma cells were found to express integrins at a higher level than normal ovarian cells [35]. Thus, integrins can be targeted in the treatment of ovarian carcinoma. Our *in vitro* data showing inhibition of ovarian cancer adhesion and invasion have supported the potential for CN as a possible treatment of this disease. However, an animal model was needed to more closely mimic the interactions between cancer cells and the environment. We chose to use the xenograft model for studying ovarian carcinoma that was established by Hamilton et al. [15]; this model allows both tumor cells and treatment to be administered by intraperitoneal injection. The spread of ovarian cancer was observed grossly in our animal model and the level of angiogenesis was quantitated immunohistochemically (factor VIII staining) to determine whether CN had an inhibitory effect on angiogenesis.

In the in vivo studies, image analysis of factor VIII-related antigen detected significantly greater microvessel areas in tissues from control than from CN-treated animals. The findings indicate that the level of angiogenesis was dramatically decreased in CN-treated animals. Moreover, the tumor burden in some of the CN-treated mice was so greatly decreased as to provide no tumor sites for angiogenic study. Thus, the area measurements (pixels) in several of these fields was quantitated to be zero. Gasparini et al. [36] established the term 'hot spot' to describe peripheral areas of the tumor that show greatest microvessel concentration. The use of image analysis for quantitation of microvessel density was found to show less variation than simple manual counts of microvessel density [37]. Although this quantitative method has been used frequently for prognostic purposes [27, 38], ours is one of the first studies to use this technology for quantitating experimental data.

CN is a good candidate for therapeutic use in ovarian cancer because it can inhibit sever-

al steps of cancer progression. Unlike other cancers, which progress by the circulatory and lymphatic systems, ovarian cancer is limited to the peritoneum. Intraperitoneal administration of CN facilitates directing its antitumor activity specifically to tumors localized in the peritoneal cavity. Thus, our investigations have demonstrated that it is feasible to design a delivery system for CN that targets potential sites of tumor growth.

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