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Antiangiogenic Therapy with Human Apolipoprotein(a) Kringle V and Paclitaxel in a Human Ovarian Cancer Mouse Model[1,2]

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Abstract

INTRODUCTION: The present study compared the effect of combination therapy using human apolipoprotein(a) kringle V (rhLK8) to conventional chemotherapy with paclitaxel for human ovarian carcinoma producing high or low levels of vascular endothelial growth factor (VEGF). *MATERIALS AND METHODS:* Human ovarian carcinoma cells producing high (SKOV3ip1) or low (HeyA8) levels of VEGF were implanted into the peritoneal cavity of female nude mice. Seven days later, mice were randomized into four groups: control (vehicle), paclitaxel [5 mg/kg, weekly intraperitoneal (i.p.) injection], rhLK8 (50 mg/kg, daily i.p. injection), or the combination of paclitaxel and rhLK8. Mice were treated for 4 weeks and examined by necropsy. *RESULTS:* In mice implanted with SKOV3ip1 cells, rhLK8 treatment had no significant effect on tumor incidence or the volume of ascites but induced a significant decrease in tumor weight compared with control mice. Paclitaxel significantly reduced tumor weight and ascites volume, and combination treatment with paclitaxel and rhLK8 had an additive therapeutic effect. Similarly, in HeyA8 mice, the effect of combination treatment on tumor weight and tumor incidence was statistically significantly greater than that of paclitaxel or rhLK8 alone. Immunohistochemical analysis showed a significant decrease in microvessel density and a marked increase of apoptosis in tumor and tumor-associated endothelial cells in response to combination treatment with paclitaxel and rhLK8. *CONCLUSION:* Collectively,

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these results suggest that antiangiogenic therapy with rhLK8 in combination with taxane-based conventional chemotherapy could be effective for the treatment of ovarian carcinomas, regardless of VEGF status.

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Introduction

Ovarian cancer is the leading cause of death from gynecological malignancies [1]. Each year, more than 190,000 new cases of ovarian cancer are diagnosed worldwide, which accounts for approximately 4% of all cancers diagnosed in women [2]. Efforts to develop screening or diagnostic tools for early detection of ovarian cancer have not been successful; therefore, a significant number of patients are diagnosed in advanced stages, requiring cytoreductive and systemic therapies such as palliative surgery and chemotherapy, respectively. However, despite an initial favorable response to chemotherapy [3,4], the heterogeneity and genetic instability of ovarian cancer cells [5] often leads to the development of drug resistance [6,7], resulting in increased cancer-related mortality. The prognosis of patients with advanced ovarian cancer has not improved over the last few decades [8], and the development of novel therapeutic strategies is therefore of critical importance [9]. Targeting relatively more homogenous and genetically stable host organ microenvironments is a new strategy that was introduced to overcome the drug resistance of tumor cells and produce better therapeutic outcomes [10,11]. The establishment of a vascular network supplying oxygen and nutrients to tumor cells is one of the most important and critical steps of tumor progression [12]. Consequently, several clinical trials have tested the efficacy of antiangiogenic molecules for blocking the interaction between tumor cells and host factors for angiogenesis, but no significant improvement has been achieved regarding the prognosis of ovarian cancer [2,13].

Kringle domains are found in many proteins with a diverse array of functions including growth factors and proteases of the blood coagulation and fibrinolysis pathways [14]. Several kringle domains in these proteins have been identified as inhibitors of angiogenesis, even though their parental proteins are not involved in angiogenesis. One of these molecules, angiostatin, includes the first three (or four) kringle domains of human plasminogen and has shown inhibitory effects on angiogenesis *in vitro* and tumor growth *in vivo* in preclinical settings [15]; however, in clinical trials, angiostatin did not show significant anticancer effects or improve clinical outcomes [16]. Human apolipoprotein(a) (apo(a)) also consists of tandemly repeated kringle domains homologous to plasminogen kringle IV (KIV), a single copy plasminogen kringle V homolog (KV), and an inactive protease domain. Previously, we demonstrated that the KIV_9, KIV_{10}, and KV domains of human apo(a), called LK68, inhibit angiogenesis and tumor growth [17]. In addition, recombinant human apo(a) KV (referred to as rhLK8) also showed antiangiogenic activity that was almost equivalent to that of LK68 [18]. Recently, we showed the therapeutic efficacy of targeting tumor-associated vasculature with rhLK8 in experimental primary and metastatic (bone) prostate carcinoma animal models [19], and on the basis of the results of the preclinical study, rhLK8 has been successfully translated into the phase I clinical trials. Studies determining the indication of the treatment are being expanded.

In this study, we examined the biologic effect of human apo(a) KV (rhLK8) on human ovarian cancer cells growing in the peritoneal cavity of female nude mice. Furthermore, we examined the antiangiogenic mechanism of action of rhLK8 and showed that combination treatment with human apo(a) KV and paclitaxel significantly inhibited tumor growth by inducing apoptosis of tumor cells and tumor-associated endothelial cells.

Materials and Methods

Human Ovarian Cancer Cell Lines and Human Apolipoprotein(a) Kringle V (rhLK8)

Two human ovarian cancer cell lines were selected for this study: SKOV3ip1, which expresses high levels of vascular endothelial growth factor (VEGF) and is associated with increased ascites formation, and HeyA8, which is characterized by low VEGF expression and no ascites formation. Those cell lines are kind gifts of Dr Isaiah J. Fidler (The University of Texas MD Anderson Cancer Center, Houston, TX) [20]. Cells were maintained in monolayer cultures in Eagle's minimal essential medium supplemented with 10% FBS (Life Technologies, Inc, Grand Island, NY), L-glutamine, pyruvate, non-essential amino acids, two-fold vitamins, and penicillin-streptomycin (Invitrogen, Carlsbad, CA) and incubated at 37°C in 5% CO_2 and 95% air.

The rhLK8 protein was expressed and purified to homogeneity as previously described [21]. Purified rhLK8 proteins were stored in buffer containing 100 mM NaCl and 150 mM L-glycine (pH 4.2). Paclitaxel (Taxol), purchased from Bristol-Myers Squibb (Princeton, NJ), was diluted in saline for intraperitoneal (i.p.) injection.

Animals

Female athymic nude mice (NCI-*nu*) were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with all current regulations and standards of the US Department of Agriculture, the US Department of Health and Human Services, and the National Institutes of Health. The mice were used in these experiments in accordance with institutional guidelines when they were 8 to 12 weeks old.

Orthotopic Implantation of Ovarian Cancer Cells in Animal Models

To establish peritoneal ovarian tumors, SKOV3ip1 or HeyA8 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. The cells were washed once in serum-free medium and resuspended in Ca^{2+}-/Mg^{2+}-free Hank's balanced salt solution. Cell viability was determined by trypan blue exclusion. Only single-cell suspensions of more than 95% viability were used for injection. SKOV3ip1 (1×10^6) or HeyA8 (2.5×10^5) cells in 200 μl of Ca^{2+}-/Mg^{2+}-free Hank's balanced salt solution were injected into the peritoneal cavity of female nude mice as previously described [22].

Therapy Experiments

Seven days after cell implantation into the peritoneal cavity, the mice were randomized into four treatment groups (n = 10 mice per group) as

follows: (1) control group, daily i.p. injection of vehicle (100 mM NaCl and 150 mM L-glycine, pH 4.2) and weekly i.p. injection of saline; (2) paclitaxel group, weekly i.p. injection of paclitaxel (5 mg/kg) and daily i. p. injection of vehicle; (3) rhLK8 group, daily i.p. injection of rhLK8 (50 mg/kg) and weekly i.p. injection of saline; and (4) combination group, daily i.p. injection of rhLK8 (50 mg/kg) and weekly i.p. injection of paclitaxel (5 mg/kg). Treatments were continued for 4 weeks.

Necropsy and Preparation of Tissues

After 4 weeks of treatment, mice were killed by CO_2 inhalation and examined by necropsy. Body weight, tumor incidence, tumor weight, and volume of ascites were recorded. Tumor tissues were embedded in OCT compound (Miles, Inc, Elkhart, IN) and rapidly frozen in liquid nitrogen or fixed in 10% buffered formalin for 24 hours and processed for paraffin blocks.

Immunohistochemical Analyses of Proliferating Cell Nuclear Antigen, Microvessel Density (CD31/platelet endothelial cell adhesion molecule-1 (PECAM-1)), Bcl-2, Bax, and VEGF

Paraffin-embedded tissues were used for identification of proliferating cell nuclear antigen (PCNA)–positive cells as previously described [23]. Frozen tissues used for identification of CD31/PECAM-1 were sectioned (8-10 μm) and fixed in cold acetone. Immunohistochemistry (IHC) procedures for PCNA and CD31/PECAM-1 were performed, and all antibodies and reagents for IHC were purchased from sources exactly as described previously [24]. Control samples exposed to secondary antibody alone showed no specific staining.

For immunohistochemical staining of Bcl-2, Bax, and VEGF, paraffin sections (4-6 μm thick) were mounted on positively charged Superfrost slides (Fisher Scientific, Co, Houston, TX) and dried overnight. Sections were deparaffinized in xylene, dehydrated with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H_2O (vol/vol)], rehydrated in phosphate-buffered saline (PBS; pH 7.5), and then microwaved for 5 minutes to improve "antigen retrieval." The slides were rinsed twice with PBS, and endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in PBS for 12 minutes. Nonspecific reactions were blocked by incubating the sections in a solution containing 5% normal horse serum and 1% normal goat serum for 20 minutes at room temperature (RT). Then, the slides were incubated overnight at 4°C with a 1:50 dilution of polyclonal antibodies against Bcl-2, Bax, or VEGF (Santa Cruz Biotechnology, Dallas, TX). The samples were then rinsed three times with PBS and incubated in HRP-conjugated goat anti-rabbit IgG at the appropriate dilutions for 60 minutes at RT. After rinsing with PBS, the slides were incubated for 5 minutes with DAB (Life Technologies, Inc), rinsed with distilled water, counterstained with Gill's hematoxylin for 1 minute, and mounted with Universal Mount (Life Technologies, Inc).

For quantification of PCNA expression, the number of positive cells was quantified in 10 random fields at ×200 magnification. To quantify mean vessel density, 10 random fields at ×100 magnification were examined for each tumor, and the microvessels within those fields were counted. A single microvessel was defined as a discrete cluster of cells stained positive for CD31 and the presence of lumen [25].

Immunofluorescence Double Staining for CD31 (Endothelial Cells) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Apoptotic Cells)

TUNEL assay was performed following CD31/PECAM-1 immunofluorescent staining as described previously [26]. The TUNEL assay was performed using a commercially available apoptosis detection kit (Promega, Madison, WI) with the following modifications. Samples were fixed with 4% paraformaldehyde for 10 minutes at RT, washed twice with PBS for 5 minutes, and then incubated with 0.2% Triton X-100 for 15 minutes at RT. After two 5-minute washes with PBS, the samples were incubated with equilibration buffer (from kit) for 10 minutes at RT. The equilibration buffer was drained, and the reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase (TdT) enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 hour in the dark. The reaction was terminated by immersing the samples in 2 × SSC for 15 minutes. Samples were washed three times for 5 minutes to remove unincorporated fluorescein-labeled deoxyuridine triphosphate (dUTP). The samples were incubated with 300 μg/ml Hoechst 33342 for 10 minutes at RT. Fluorescence bleaching was minimized by treating slides with a ProLong anti-fade reagent (Life Technologies, Inc). The apoptotic index of tumor-associated endothelial cells was determined by co-localization of CD31 and TUNEL staining. Endothelial cells and DNA fragmentation in apoptotic cells were identified by red and green fluorescence, respectively, and apoptotic endothelial cells were identified by yellow fluorescence within the nucleus. Apoptotic tumor cells and tumor-associated endothelial cells were identified and counted in five random fields at ×400. Images were captured by an Olympus BX-51 microscope (Olympus America, Inc, Center Valley, PA).

Statistical Analyses

Tumor incidence, tumor weight, ascites volume (Mann-Whitney *U* test), the number of PCNA-positive cells, and microvessel density (MVD; CD31/PECAM-1) (unpaired Student's *t* test) were compared in each treatment group. All values are expressed as means ± SD except where indicated.

Results

Effects of rhLK8 on the Tumor Growth

We determined the biologic effects of rhLK8 and paclitaxel on the growth of SKOV3ip1 human ovarian cancer cells producing high levels of VEGF injected into the peritoneal cavity of female nude mice. Paclitaxel significantly reduced tumor weight [0.04 g (0-0.2 g) *vs* 0.98 g (0.66-1.63g); median (range), $P < .01$)] and ascites [0.1 ml (0-0.2 ml) *vs* 0.9 ml (0.5-1.6 ml); median (range), $P < .05$] compared to control mice. No significant differences in tumor incidence or ascites volume were detected between control mice and mice treated with rhLK8 alone; however, rhLK8 significantly decreased tumor weight compared to the control [Table 1; 0.65 g (0.01-1.3 g) *vs* 0.98 g (0.66-1.63 g); median (range), $P < .05$]. Combination treatment with paclitaxel and rhLK8 had an additive effect on reducing tumor weight [control group 0.98 g (0.66-1.63 g) *vs* combination group 0.01 g (0-0.14 g); median (range), $P < .01$)] and the volume of ascites [control group 0.9 ml (0.5-1.6 ml) *vs* 0 ml (0-0.2 ml); median (range), $P < .05$].

The biologic effects of rhLK8 were also examined in mice injected with HeyA8 human ovarian cancer cells producing low levels of VEGF (Table 1). In these mice, tumor weight was significantly reduced by treatment with paclitaxel or rhLK8 alone compared to that in control mice [2.1 g (0-3.6 g) *vs* 4.0 g (0.2-7.2 g), $P < .05$ and 1.0 g (0-6.0 g) *vs* 4.0 g (0.2-7.2 g), $P < .05$, respectively]. Combination treatment with paclitaxel and rhLK8 had a significant and synergistic effect on decreasing tumor incidence (55.6 % *vs* 100%, $P < .05$) and tumor weight [0.3 g (0-2.4 g) *vs* 4.0 g (0.2-7.2 g), $P < .01$]. No substantial

Table 1. Therapy of Human Ovarian Cancers Growing in the Peritoneal Cavity of Female Nude Mice.

	Treatment Groups	Tumor Incidence	Tumor Weight (g) [Median (range)]	Ascites (ml) [Median (range)]
SKOV3ip1	Control	10/10	0.98 (0.66-1.63)	0.9 (0.5-1.6)
	Paclitaxel	9/10	0.04 (0-0.20)**	0.1(0-0.2)*
	rhLK8	10/10	0.65 (0.01-1.30)*	0.5 (0-1.9)
	rhLK8 + paclitaxel	6/10	0.01 (0-0.14)**	0 (0-0.2)*
HeyA8	Control	10/10	4.0 (0.2-7.2)	NA
	Paclitaxel	8/10	2.1 (0-3.6)*	NA
	rhLK8	7/10	1.0 (0-6.0)*	NA
	rhLK8 + paclitaxel	5/9*	0.3 (0-2.4)**	NA

Note: 1×10^6 SKOV3ip1 cells or 2.5×10^5 HeyA8 cells were implanted into the peritoneum of female nude mice. Seven days later, treatment with vehicle, paclitaxel (5 mg/kg, weekly i.p. injection), and rhLK8 (50 mg/kg, daily i.p. injection) alone or in combination with paclitaxel was started and continued for 4 weeks. Tumor incidence, tumor weight, and ascites volume were determined. *$P < .05$; **$P < .01$. NA, not applicable.

Table 2. Cellular Proliferation, Mean Vessel Density, and Apoptosis of Tumor-Associated Endothelial Cells in Human Ovarian Cancers Growing in the Peritoneal Cavity of Female Nude Mice.

	Treatment Groups	PCNA (Mean ± SD)	CD31 (Mean ± SD)	$TUNEL^+CD31^+$ (Mean ± SD)
SKOV3ip1	Control	108.4 ± 24.7	84.0 ± 27.5	0.6 ± 1.0
	Paclitaxel	64.4 ± 17.3**	73.1 ± 20.4	4.0 ± 2.1*
	rhLK8	74.0 ± 17.6	44.0 ± 9.7**	11.7 ± 4.0**
	rhLK8 + paclitaxel	41.0 ± 12.8**	29.4 ± 5.7**	31.3 ± 9.4***
HeyA8	Control	98.4 ± 16.1	57.1 ± 18.5	0.2 ± 0.4
	Paclitaxel	88.6 ± 16.9	40.0 ± 15.7*	2.7 ± 1.6*
	rhLK8	76.1 ± 20.0	27.0 ± 6.1**	7.3 ± 3.4**
	rhLK8 + paclitaxel	55.9 ± 14.2**	14.3 ± 5.0***	26.4 ± 10.2***

Note: After 4 weeks of treatment, SKOV3ip1 or HeyA8 tumors were harvested and stained with PCNA, CD31/PECAM, and/or TUNEL. For quantification of PCNA, mean vessel density, and apoptotic endothelial cells, the number of positive cells was quantified in 10 random fields at ×200 magnification and ×100 magnification and five random fields at ×400, respectively. *$P < .05$; **$P < .01$; ***$P < .001$.

differences in the body weight of mice were observed among treatment groups (data not shown).

VEGF levels were ~10-fold higher in SKOV3ip1 cells than in HeyA8 cells. Treatment of cells with rhLK8 for 48 hours had no significant effect on VEGF levels in SKOV3ip1 or in HeyA8 cells (Figure W1).

Determination of Cellular Proliferation and MVD

Cellular proliferative index was determined according to the number of PCNA-positive cells, and MVD was determined by the number of CD31-positive cells. The number of PCNA-positive cells was significantly lower in paclitaxel-treated SKOV3ip1 tumors than in control mice (64.4 ± 17.3 *vs* 108.4 ± 24.7, $P < .01$), whereas no significant reduction was observed in response to rhLK8 treatment (74.0 ± 17.6 *vs* 108.4 ± 24.7, $P > .05$). The most significant decrease in the number of PCNA-positive cells was observed in SKOV3ip1 tumors treated with the combination of paclitaxel and rhLK8 (41.0 ± 12.8 *vs* 108.4 ± 24.7, $P < .01$; Table 2 and Figure 1*A*). In HeyA8 tumors, treatment with paclitaxel or rhLK8 alone did not significantly decrease the number of PCNA-positive cells (88.6 ± 16.9 *vs* 98.4 ± 16.1, $P > .05$ and 76.1 ± 20.0 *vs* 98.4 ± 16.1, $P > .05$, respectively); however, combination treatment significantly reduced the number of PCNA-positive cells (55.9 ± 14.2 *vs* 98.4 ± 16.1, $P < .01$; Table 2 and Figure 1*B*).

No significant differences in MVD were detected between control and paclitaxel-treated SKOV3ip1 tumors (84.0 ± 27.5 *vs* 73.1 ± 20.4, $P > .05$); however, treatment with rhLK8 alone and, in particular, the combination of rhLK8 and paclitaxel significantly decreased MVD in SKOV3ip1 tumors as compared with the controls (44.0 ± 9.7 *vs* 84.0 ± 27.5, $P < .01$ and 29.4 ± 5.7 *vs* 84.0 ± 27.5, $P < 0.01$, respectively; Table 2 and Figure 2*A*). In HeyA8 tumors, MVD was significantly reduced by treatment with paclitaxel compared with the control group (40.0 ± 15.7 *vs* 57.1 ± 18.5, $P < .05$) and to a greater extent with rhLK8 alone (27.0 ± 6.1 *vs* 57.1 ± 18.5, $P < .01$) or the combination of paclitaxel and rhLK8 (14.3 ± 5.0 *vs* 57.1 ± 18.5, $P < .001$; Table 2 and Figure 2*B*).

Determination of Apoptosis of Tumor Cells and Tumor-Associated Endothelial Cells by Co-Localization of CD31 and TUNEL

Immunofluorescence double staining of CD31 (red) and TUNEL (green) was performed to evaluate apoptosis of tumor cells and tumor-associated endothelial cells in response to the different treatments. Apoptosis of endothelial cells is indicated by co-localization, detected by a yellow signal. In SKOV3ip1 tumors (Table 2 and Figure 3*A*), few tumor cells or tumor-associated endothelial cells were apoptotic in the control group. Paclitaxel treatment significantly induced apoptosis in tumor-associated endothelial cells compared with the control group (4.0 ± 2.1 *vs* 0.6 ± 1.0, $P < .05$). A more significant increase in apoptosis was induced by rhLK8 alone (11.7 ± 4.0 *vs* 0.6 ± 1.0; $P < .01$), and the combination of the two drugs enhanced this effect (31.3 ± 9.4 *vs* 0.6 ± 1.0, $P < .001$). A similar trend was observed in HeyA8 tumors (Table 2 and Figure 3*B*), in which paclitaxel significantly induced apoptosis compared to the control group (2.7 ± 1.6 *vs* 0.2 ± 0.4, $P < .05$), and the effect was enhanced by rhLK8 (7.3 ± 3.4 *vs* 0.2 ± 0.4, $P < .01$) or the combination of the two drugs (26.4 ± 10.2 *vs* 0.2 ± 0.4, $P < .001$). In the SKOV3ip1 and HeyA8 tumor models, apoptosis of tumor cells was induced only in the paclitaxel treatment group and not in the rhLK8 treatment group, whereas the combination of paclitaxel and rhLK8 intensified the apoptosis of tumor cells (Figure 3). On the basis of the findings by Oltval and colleagues [27] suggesting that the Bax/Bcl-2 ratio governs the sensitivity of cells to apoptotic stimuli, we analyzed the expression of Bax and Bcl-2 proteins in the tumor tissues and observed that there were no significant changes in Bcl-2 expression in the tumor tissues from control and treatment groups, but the expression of BAX was significantly increased in the tumor tissues from combination treatment group of mice (Figure W2).

To investigate the effects of rhLK8 or paclitaxel treatment, as a single agent or combination of two drugs, on the expression of VEGF in the tumor tissues, immunohistochemical staining of VEGF was performed. VEGF expression in SKOV3ip1 tumors was significantly higher than that in HeyA8 tumors, compared to tumors of control groups (Figure W3). Treatment of mice with either paclitaxel or rhLK8 did not significantly alter the expression of VEGF; however, expression of VEGF in tumors of HeyA8 was slightly increased in tumors of mice treated with either paclitaxel or rhLK8 (Figure W3*B*). Treatment with the combination of paclitaxel and rhLK8 significantly decreased the expression of VEGF (Figure W3*B*).

Discussion

Ovarian tumors possess a rich vascular network that is highly dependent on VEGF-mediated angiogenesis [28,29]. Therefore, many angiogenesis inhibitors have been evaluated in the preclinical and clinical settings for the treatment of ovarian carcinoma [30]. One of the most extensively studied vascular targeting molecules is bevacizumab (Avastin), which neutralizes circulating VEGF and suppresses angiogenesis [31]. Recent phase III clinical trials in first-line ovarian cancers showed that bevacizumab prolonged progression-free survival when administered in combination with chemotherapy [32]. However, the effect of anti-

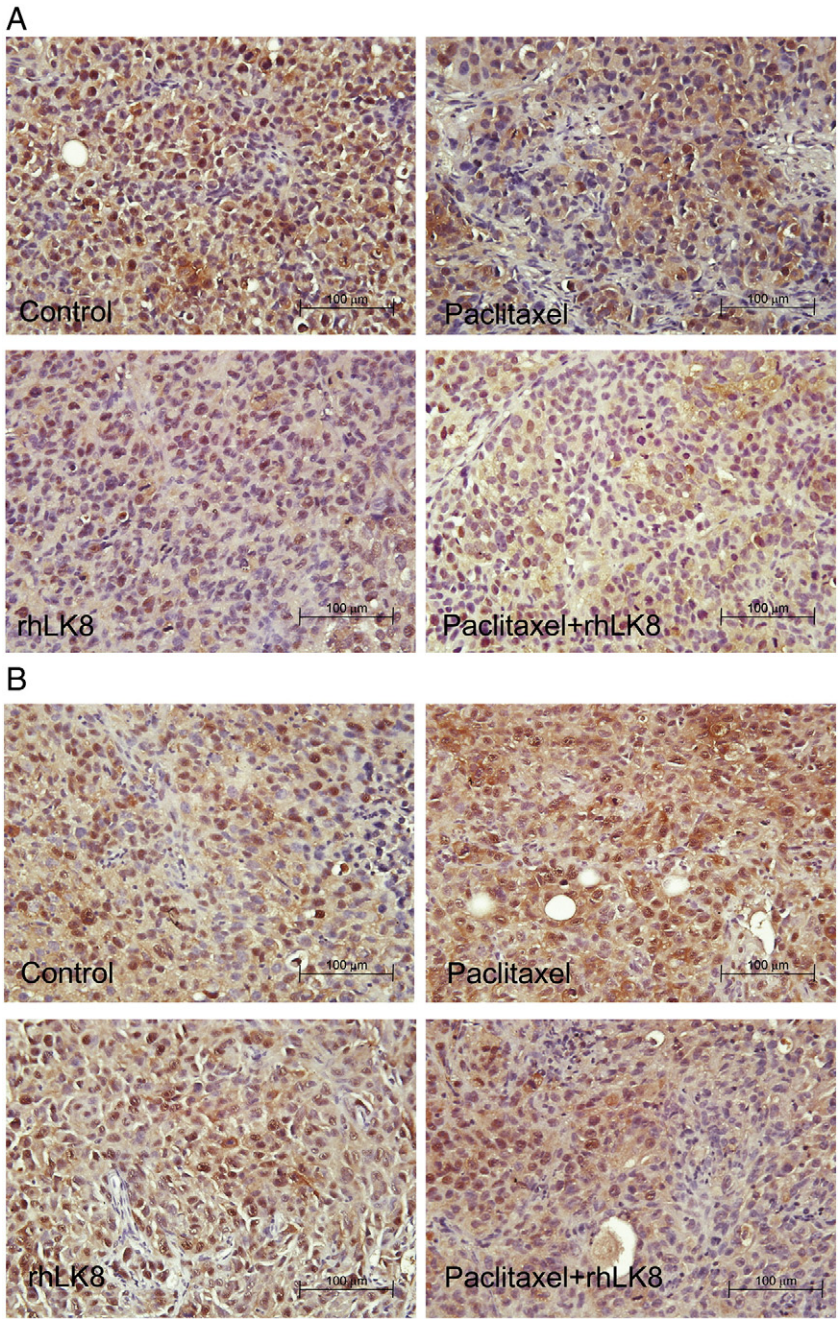

Figure 1. Analysis of cellular proliferation. (A) In SKOV3ip1 tumors, paclitaxel significantly decreased the number of proliferative (PCNA-positive) cells, and this effect was enhanced in response to combination treatment with paclitaxel and rhLK8. (B) In HeyA8 tumors, treatment with paclitaxel alone or rhLK8 alone did not change the cellular proliferative index (PCNA-positive cells), but combination treatment with paclitaxel and rhLK8 significantly decreased the number of PCNA-positive cells.

VEGF therapy on overall survival is limited and it is often associated with several clinical toxicities [33,34]. Moreover, tumor cells can escape from prolonged anti-VEGF therapy by producing other proangiogenic factors [35]. Therefore, the development of antiangiogenic drugs that are effective independent of the VEGF status of tumors is critical.

Our results clearly showed the efficacy of antiangiogenic therapy with rhLK8 in combination with paclitaxel on the proliferation of human ovarian carcinoma cells producing high or low levels of VEGF in a xenograft mouse model. We examined two human ovarian cancer cell lines with significantly different VEGF levels and expected to find differences in the biologic activity of the VEGF axis. Tumors derived from SKOV3ip1 cells grew relatively slower, produced higher levels of VEGF, induced the development of ascites, and showed higher MVD, whereas HeyA8 cells formed larger tumors with lower VEGF expression levels that did not produce ascites and showed lower MVD. Treatment with paclitaxel or rhLK8 as a single agent significantly reduced tumor size but not tumor incidence in both models. Immunohistochemical analysis of tumor specimens revealed that paclitaxel treatment inhibited tumor cell proliferation, whereas rhLK8 showed minimal effects; however, rhLK8 treatment significantly induced apoptosis of tumor-associated endothelial cells and decreased MVD, whereas paclitaxel had no or a minimal effect. Although rhLK8 significantly reduced tumor size in a limited period of time (~4 weeks) by inducing apoptosis of tumor-associated

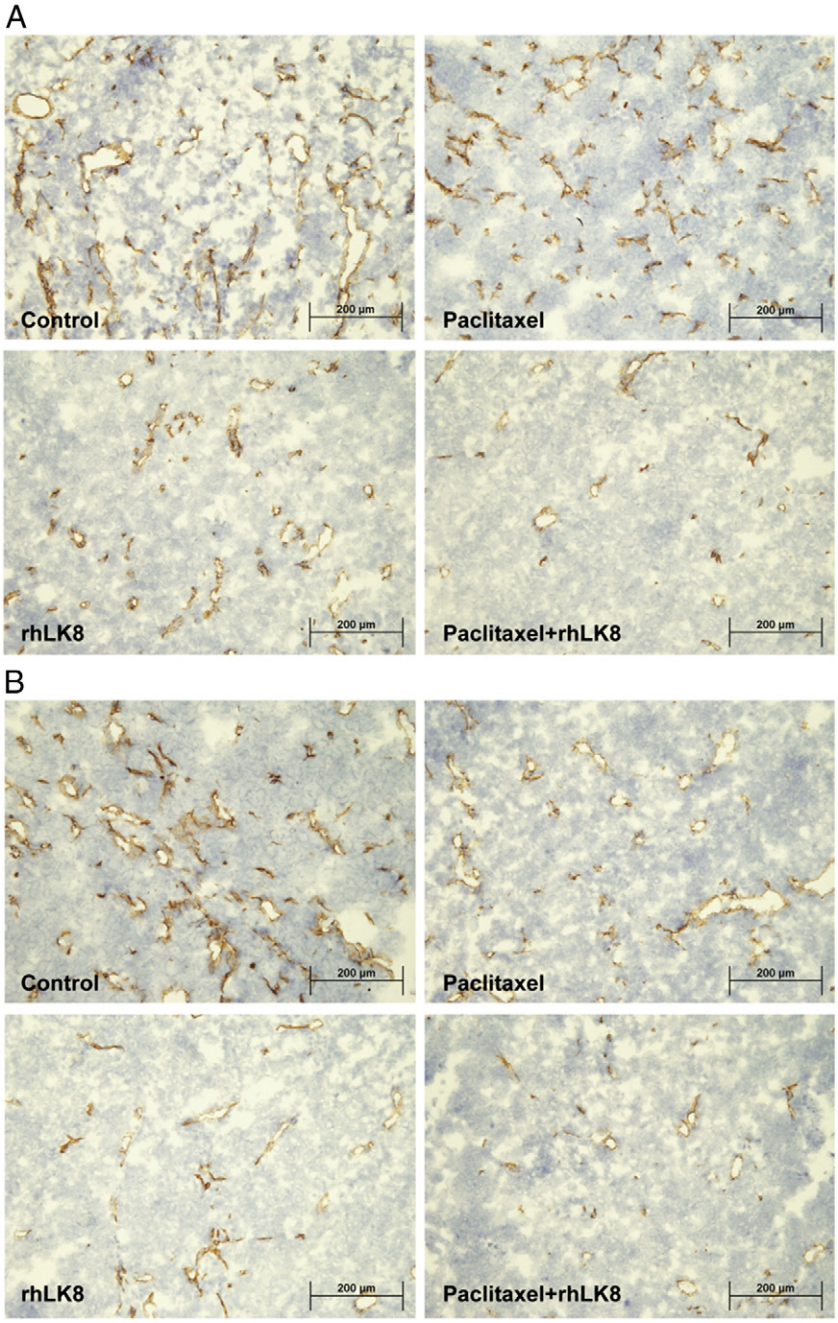

Figure 2. MVD of tumors by immunohistochemical staining of CD31. (A) In SKOV3ip1 tumors, rhLK8 significantly reduced the number of CD31-positive cells, and this effect was enhanced by combination treatment with rhLK8 and paclitaxel. (B) In HeyA8 tumors, treatment with paclitaxel alone significantly decreased MVD as determined by the number of CD31-positive cells, and this effect was intensified by rhLK8 treatment and most significantly by the combination of paclitaxel and rhLK8.

endothelial cells, leading to the induction of apoptosis of nearby tumor cells nourished by the same vasculature, it did not affect tumor cell proliferation. These findings suggest that the cytostatic nature of angiogenesis inhibitors, including rhLK8, may limit their ability to control the growth of cancer cells, and combination therapy with chemotherapeutic agents may be necessary to enhance their therapeutic efficacy and to prolong the median survival of patients with ovarian cancer. In this study, we found that antiangiogenic and antitumor efficacy was dramatically improved in mice treated with the combination of paclitaxel and rhLK8. Our results are in agreement with an increasing body of work demonstrating that the combination of angiogenesis inhibitors with chemotherapeutic drugs significantly improves treatment outcomes compared to single agent therapy. Tumor blood vessels are irregular, dilated, tortuous, and leaky, which leads to elevated tumor interstitial fluid pressure and thus inefficient delivery of chemotherapeutic agents [36]. Antiangiogenic therapy may induce the transient normalization of tumor vasculature, which enhances the delivery of chemotherapeutic agents such as paclitaxel by decreasing interstitial pressure, leading to an increase in therapeutic efficacy [37]. In addition, rhLK8 may attenuate the survival pathway of tumor-associated endothelial cells, which makes proliferating tumor-associated endothelial cells more sensitive to anticycling drug,

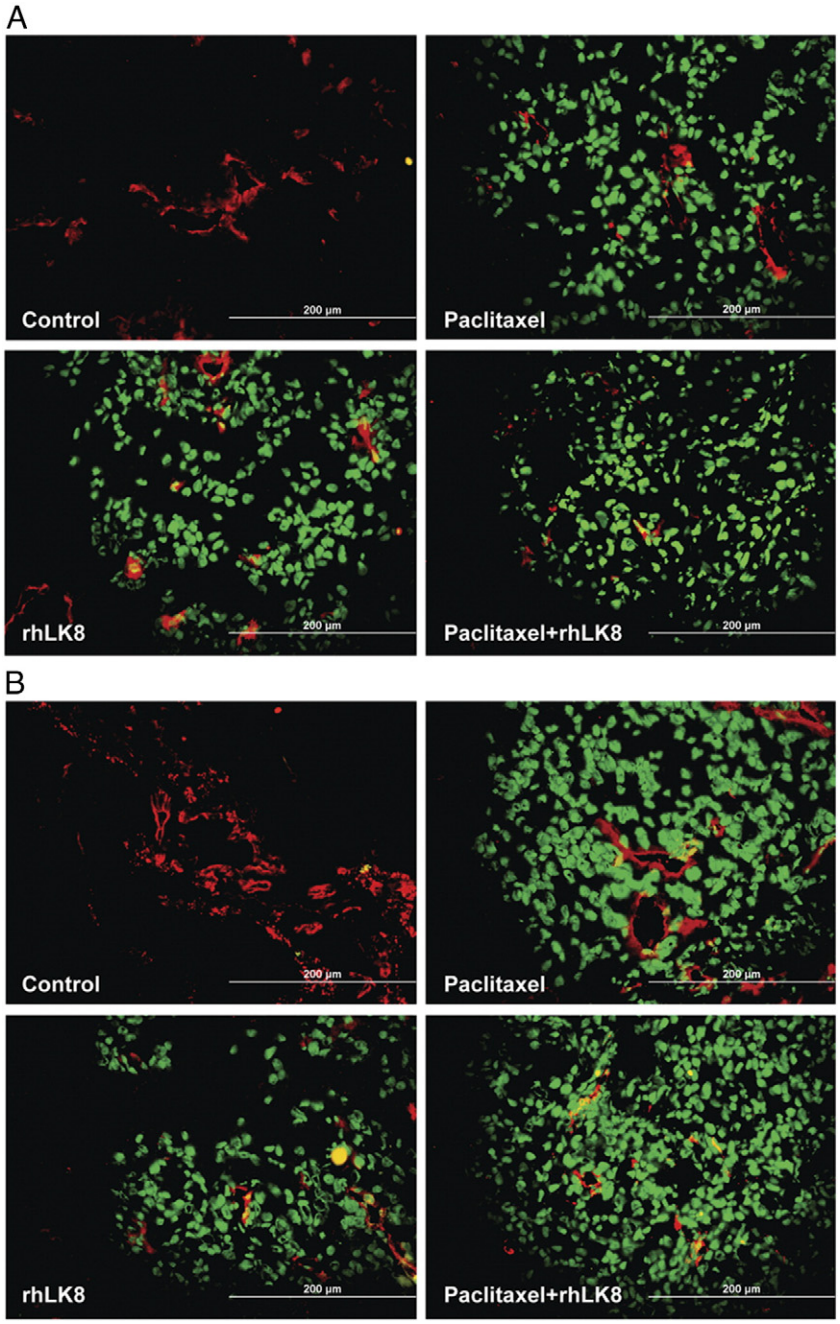

Figure 3. Apoptosis of tumor cells and tumor-associated endothelial cells. Tumor specimens from (A) SKOV3ip1 or (B) HeyA8 tumors were subjected to immunofluorescence double labeling, and the co-localization of signals for CD31 and TUNEL was determined. Treatment with paclitaxel alone significantly induced apoptotic (TUNEL-positive, green) tumor cells and tumor-associated endothelial cells (TUNEL-positive and CD31-positive, yellow), and this effect was intensified by treatment with rhLK8 alone. Combination treatment (paclitaxel plus rhLK8) had the most significant effect on the induction of apoptosis.

paclitaxel. The improved therapeutic outcomes induced by combination therapy with rhLK8 appear not to be limited to taxane-based chemotherapy. Our preliminary data showed that combination therapy of rhLK8 with gemcitabine or irinotecan (or 5-fluorouracil) improved treatment outcomes than the corresponding treatment as a single agent in the human pancreatic and colon carcinoma animal models, respectively (Kim JS et al., unpublished data). In this context, combination of rhLK8 with other chemotherapeutic agents such as carboplatin or cisplatin, which have been regarded as the primary treatment option of advanced ovarian cancer together with paclitaxel, was also expected to show improved treatment outcomes, although appropriate preclinical and/or clinical evaluation of the combination therapy will be critically required.

Paclitaxel significantly reduced the volume of ascites in SKOV3ip1 tumor–bearing mice but rhLK8 alone did not. However, the effect of rhLK8 on decreasing MVD was more significant than that of paclitaxel. These results are in agreement with prior findings suggesting that ascites formation may depend on the levels of VEGF (originally referred to as vascular permeability factor) produced by tumor cells rather than MVD [38], because paclitaxel-treated

tumors were smaller (and displayed a lower tumor burden) than those treated with rhLK8 alone. Interestingly, rhLK8 as a single agent significantly reduced tumor size, and this effect was more pronounced in HeyA8 tumors producing low levels of VEGF. rhLK8 appears not to target tumor cells but inhibits activated endothelial cells. Therefore, antitumor efficacy of rhLK8 was independent on the VEGF expression of the corresponding tumor cells. Because SKOV3ip1 produced the profound amount of biologically active VEGF, they have more biologic redundancy in the survival of tumor-associated endothelial cells than HeyA8, which depends on more tight and narrower angiogenesis activity. Therefore, when rhLK8 inhibits angiogenesis of tumor-associated vasculature, there would be more impact on the HeyA8 tumors. This may explain the synergistic therapeutic effect of rhLK8 on HeyA8 cells. Collectively, these results suggest that VEGF may not be a determinant of the response of certain cancers to antiangiogenic therapy with rhLK8.

In this study, expression of VEGF was increased in HeyA8 tumors of mice treated with paclitaxel or rhLK8. This may be from the local hypoxic condition induced by impaired tumor vasculature caused by either destruction of proliferating tumor-associated endothelial cells by paclitaxel or suppressed angiogenesis by rhLK8. Because intrinsic level of expression in SKOV3ip1 tumors was high, it was not altered by the local hypoxia induced by the treatment with either paclitaxel or rhLK8. Decreased expression of VEGF in tumors of mice treated by the combination of paclitaxel and rhLK8 may reflect the decreased biologic activity or, further, viability of tumor cells from additive or synergistic effects on the induction of the apoptosis of tumor-associated endothelial cells.

Previously, we showed that combination treatment with paclitaxel and rhLK8 could be an effective therapy for prostate cancer metastatic to the bone [19], as well as other solid tumors including colorectal carcinoma, pancreatic carcinoma, renal cell carcinoma, and melanoma (data not shown); however, macroscopic (tumor incidence and tumor size) or microscopic (cellular proliferation, MVD, or apoptosis) responses to therapy with paclitaxel and rhLK8 were not observed in orthotopic animal models of human lung cancer, PC14 cells, which produce high levels of VEGF, and pleural effusion (Kim JS et al., unpublished data).

The mechanisms mediating the different responses to rhLK8 treatment between tumors growing in different organs are not clear. One possible explanation is that rhLK8 may have a differential effect on the biologic properties of tumor-associated endothelial cells, because the specific features of endothelial cells have been reported to be organ-dependent [39,40]. Furthermore, the interaction of rhLK8 with glucose-regulated protein 78 on the endothelial cell surface plays an important role in rhLK8-mediated apoptosis of endothelial cells (Ahn JH et al., unpublished data), although the impact of this interaction in the endothelial cells of certain organs or that of interactions with other target protein(s) or receptor(s) on the organ-specific therapeutic outcomes mediated by rhLK8 remains unclear. Moreover, tumor cell features such as the activation of some oncogenes and interactions with components of the tumor microenvironment, such as immune cells, may affect the angiogenic phenotype of the tumors [41]. Therefore, the effects of rhLK8 on those factors cannot be ruled out. This possibility is supported by the finding that plasminogen kringle 5, which has significant sequence homology with rhLK8, can exert its antitumor activity either by inhibiting the recruitment of tumor-associated macrophages or by promoting the recruitment of neutrophils or NKT lymphocytes [42,43].

In conclusion, our results suggest that antiangiogenic therapy with rhLK8 in combination with taxane-based conventional chemotherapy could be a promising therapeutic approach to the treatment of patients with ovarian cancer. Furthermore, the level of VEGF expressed or produced by tumor cells may not be the absolute determinant as the indication of antivascular therapy with rhLK8. Human apo(a) KV, rhLK8, has recently entered phase I clinical trials in patients with cancer. The safety and therapeutic outcomes of the combination of rhLK8 with conventional chemotherapy should also be assessed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2014.04.005.

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