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INFLUENCE OF ANGIOSTATIN AND THALIDOMIDE ON LYMPHANGIOGENESIS

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ABSTRACT

Malignant cancers commonly invade locally followed by spread through venous or lymphatic channels or both to distant sites. Hemangiogenesis and its relation to tumor growth and metastasis have been extensively studied. However, the role played by lymphangiogenesis in growth and metastasis of cancer has been largely neglected until just recently. Inhibition of lymphangiogenesis, as compared to inhibition of hemangiogenesis, may provide new insights into the mechanisms of cancer metastasis. The current study was designed to examine the in vitro *effect of two commonly used inhibitors of hemangiogenesis, angiostatin and thalidomide, on the growth and proliferation of lymphatic endothelial cells isolated from pig thoracic ducts. We first isolated and characterized the lymphatic endothelial (LE) cells using specific markers for VEGFR3 and LYVE-1. The experimental results showed that treatment of the LE cells with these two drugs resulted in a decrease in the rate of cell proliferation in a dose-dependent manner as assessed by MTT assays. Cell migration rate was assessed by the speed of cell migration from the scrape-wound margin, and the results showed that migration of LE cells was also significantly inhibited in a dose-dependent fashion compared to controls. Treatment with angiostatin and thalidomide both resulted in an increase in apoptosis of LE cells as assessed by Hoechst staining and flow cytometry. We conclude that both angiostatin and thalidomide are able to inhibit LE cell growth in a dose-dependent manner and that the inhibition may be through induction of apoptosis.*

Metastasis is a common feature of many malignant cancers. The route taken by cancer cells after the initial break-off from its original site could be by venous channels, lymphatic channels or both. Extensive research has been focused on the relationship of angiogenesis to growth of tumors in the past three decades. Different strategies have been developed to stop the growth and spread of cancers by restricting angiogenesis. One of these is by cutting off the blood supply which fuels the growth of tumors by inhibiting the formation of new vessels to the tumor, thus, starving the tumor cells. This approach has achieved some success. Though the scientific community has now a much better understanding of the factors regulating angiogenesis involving blood vessels, there has been much less attention to the factors regulating lymphangiogenesis, i.e., formation of new lymphatic vessels and the role of this process in regulating the growth and speed of tumors. Grossfeld, et al (1) have suggested that necrosis of tumors is related to a lack of lymphatic vessels. Achen, et al (2) have reviewed the current literature on tumor lymphangiogenesis supporting the notion that inhibiting the formation of new lymphatic vessels might also be a valuable approach to controlling the growth and spread of cancer.

Angiostatin was first isolated from serum

of EOMA by O'Reilly in 1997 (3). It is a proteolytic fragment of plasminogen which has a greater than 98% sequence homology to a 38 kDa internal fragment of plasminogen with an N-terminus beginning at amino acid 98 and a C-terminus at amino acid 440. Angiostatin comprises the first four kringle domains of the plasminogen (~85kDa). Kringle 1-3 have a potent inhibition of endothelial cell proliferation, whereas kringle 4 shows only a marginal effect (4). Previous studies under *in vitro* and *in vivo* conditions have shown that angiostatin effectively inhibits proliferation and migration of blood vessel endothelial cells and induces apoptosis. These studies also show that angiostatin has an inhibitory effect on the key steps of angiogenesis, including proliferation, migration and 'tube' formation of endothelial cells (5).

Thalidomide, on the other hand, is a sedative with 7.8-kDa protein with 70 amino acids (6). It was first developed by Grunenthal in the 1950s and used to treat morning sickness in early pregnancy, but due to its teratogenic potential, it was banned and soon withdrawn from the market. The compound is also well known for its strong anti-inflammatory and immunosuppressive potentials. Recent studies have shown that thalidomide possesses a powerful antiangiogenic effect. The finding of thalidomide's antiangiogenic action has revived therapeutic interest in this compound despite its known teratogenic effect on early embryonic development. D'Amato, et al (7) reported that angiogenesis induced by bFGF in the rabbit cornea could be inhibited by thalidomide. Kenyon, et al (8) further found that thalidomide was not only an inhibitor of angiogenesis induced by bFGF, but also inhibited angiogenesis induced by VEGF through down-regulation of TNF-α.

This study was designed to examine the potential influence of the angiogenic inhibitors angiostatin and thalidomide on the proliferation and migration of lymphatic endothelial cells, i.e., lymphangiogenesis and possible underlying mechanisms.

MATERIALS AND METHODS

Origin, Isolation and Culture of Lymphatic Endothelial Cells (9)

Lymphatic endothelial (LE) cells were obtained from the pig thoracic duct. Fresh pig thoracic ducts were obtained at the local abattoir and kept fresh until use. A segment of 10-15 cm of the thoracic duct was dissected out and cleaned of the surrounding fat. The distal end was cannulated with polythene tubing, and a 0.1% collagenase (Sigma, Type IA) solution in PBS was injected into the thoracic duct. The proximal end of the thoracic duct was ligated and placed in a humid incubator for 10 minutes at 37°C . After incubation, collagenase solution containing the detached endothelial cells was drained and centrifuged at 1,000 rpm for 10 minutes. The cell pellet was resuspended in the culture medium supplemented with 20% fetal calf serum, 100 ug/ml penicillin, 100 µg/ml streptomycin, and 78% DMEM before seeding in a culture vessel. They were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2. After 2-3 weeks of culture, LE cells formed a confluent monolayer. The second or third passage of LE cells was used for all experiments.

Characterization of LE Cells

The isolated LE cells were characterized first by the presence of both Factor VIII-related antigen, as a specific marker for all endothelial cells, and then by VEGFR-3 and LYVE-1, as specific markers for LE cells.

Factor VIII-Related Antigen (Sigma) Marker Staining for Endothelial Cells

The LE cells were grown on a glass cover slip to confluency and fixed in 95% alcohol. After washing in PBS, 10% sheep serum was added at RT for 10 min. The coverslip was first covered with antibody against Factor VIII-related antigen (1:200) and incubated at

37°C for 2 hr. This was followed by washing in PBS, the addition of the second antibody (sheep anti-rabbit IgG-FITC, 0.1mg/ml), and incubation at 37°C for a further 10 min. The coverslips were washed, mounted, and cells were observed under a fluorescent microscope.

VEGFR-3 (10) (Sigma) and LYVE-1 (11) (AngioBio), Specific Markers Stained for Lymphatic Endothelial Cells

The cells grown on coverslips were fixed with 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, for 20 min in RT. After PBS washing, the cover slip was treated with 10% sheep serum at 37°C for 30 min before the addition of the first antibody (i.e., anti-VEGFR-3, 5 µg/ml or anti-LYVE-1, 25 µg/ml) and incubated at 4°C overnight. Following PBS washing, the cells were treated with a second antibody (sheep anti-rabbit IgG-FITC, 0.1mg/ml) and incubated at 37_C for 1 hr. After washing, the cells were examined by laser confocal microscope (Zeiss).

Treatment of LE Cells by Angiostatin and Thalidomide for Assessment of Cell Migration Rate

Confluent second passage LE cells were used in all experiments. An artificial wound was introduced by a rubber policeman to create a gap of about 10mm in the middle of the confluent LE cell culture. The cells were then allowed to grow for a further 24 hr in medium containing different concentrations of angiostatin (0.5, 0.7 and 1.0 µg/ml) or thalidomide (0.5, 0.7 and 1.0 µg/ml) before analysis. Controls were cultured under identical condition but without addition of either of the two drugs. Migration rates of LE cells were determined by counting the number of cells and measuring the distance from which the cells had migrated from the wound margin.

Assessment of Cell Proliferation Rate by MTT Assay (12)

The growth rate of the cells was measured by the MTT proliferation assay (12). LE cells (5 x 10^4 cells/ml) were seeded in each well into 96-well plates. Angiostatin at various concentrations (0.5, 0.7, 1.0, 1.25 µg/ml) or thalidomide (0.3, 0.5, 0.7, 1.0, 1.5 µg/ml) were added to the culture 24 hr later. Control cultures were untreated. 24 hr later, 100 µl of 0.5 mg/ml of MTT reagent was added to each well and then incubated for 4 hr at 37°C. At the end of incubation, the formazan crystal formed was dissolved by dissolving reagent and the OD value was measured at 490 nm. Eight wells were used for each concentration, and each concentration was repeated 3 times. The results were expressed as the percentage of treated cells over the untreated cells.

Apoptotic Assay (Hoechst Staining)

0.5 ml of LE cells (5 x 10^4 cells/ml) were seeded onto the cover slip in 24-well plates and cultured for 24 hr. Designated amounts of angiostatin (0.5, 0.7, 1.0, 1.25 µg/ml) or thalidomide (0.3, 0.5, 0.7, 1.0, 1.5 µg/ml) were added to each well respectively, and cultured for a further 24 hr. The cells were then fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 for 1 h at RT. After PBS washing, the cells were stained by Hoechst staining reagent (10 µg/ml) for 10 min before examination using a fluorescent microscope.

Flow Cytometry (13)

This assay was also utilized to analyze cell apoptosis by measuring DNA content of the cells. LE cells at a concentration of 2-10x10^4 cells/ml were seeded into culture flasks and cultured for 48 hr. The culture medium was removed and replenished with medium containing different concentrations of angiostatin (1.5 and 2.0 µg /ml) or thalidomide (2.0 and 3.0 µg /ml) and cultured for 24 hr. The controls were untreated and cultured for 24 hr. Cells were trypsinized and centrifuged for 5 min (1,000 r/min) and

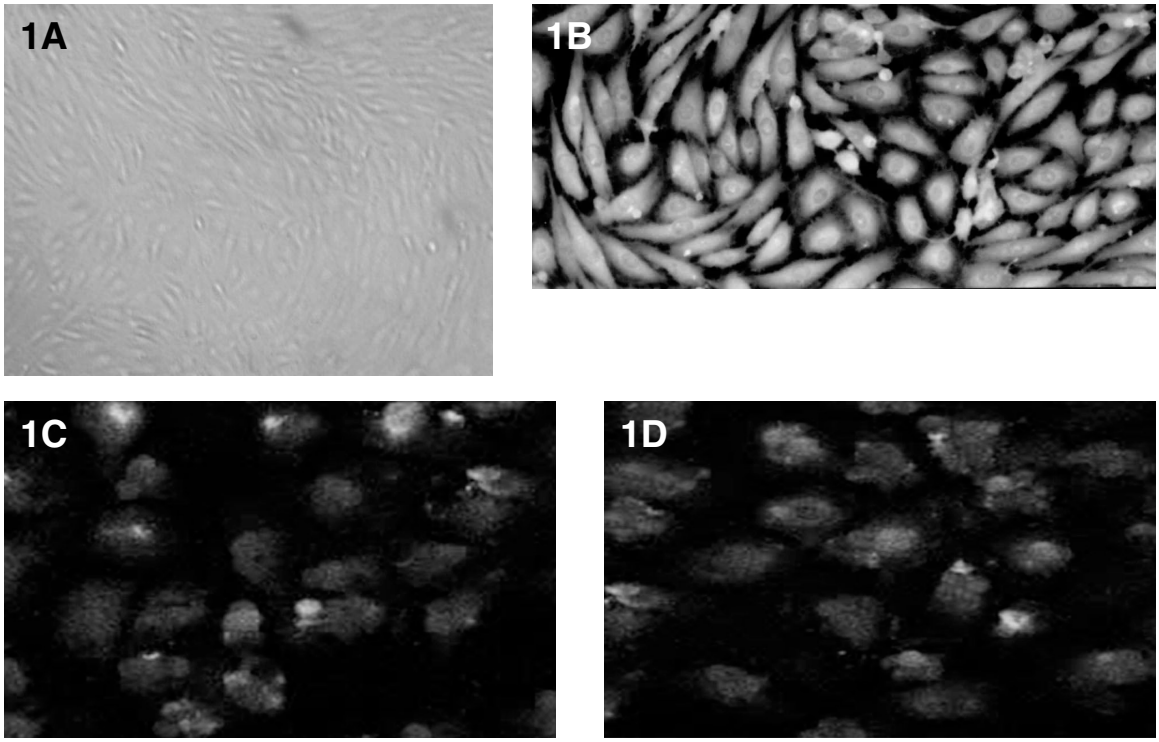

Fig. 1. (A) 2-3 week culture of procine LE cells forming a confluent monolayer. x 100. (B) LE cells are characterized by positivity to Factor VIII-related antigen. Note the intense light staining reaction in the perinuclear region. x200. (C) LE cells displaying positive staining for VEGFR-3 and (D) LYVE-1. x200.

washed. 200 µl of propidium iodide (50 µg /ml) was added, incubated for 30 min in dark at RT, and cells examined by a flow cytometer (Vantage, BD Company).

Statistical Analysis

The data were analyzed using Student-Newman-Keuls to compare the significance of difference between the control and experimental groups (Sigma Stat software). The results are displayed as mean ± standard error. P values < 0.05 and 0.01 were deemed as significant and highly significant, respectively.

RESULTS

Morphological Characteristics of LE cells

After 6-12 h in culture, LE cells began to adhere to the culture dish and formed small clumps. The cells grew quickly out from the clumps in the next several days and by 2-3 weeks, the LE cells had grown to near confluency. They exhibited typical cobblestone feature characteristic of LE cells (*Fig. 1A*). Immunofluorescent examination revealed the presence of Factor VIII-related antigen reactivity in the perinuclear region (*Fig. 1B*), which is specific to all endothelial cells. On the other hand, the results from confocal microscopy showed in addition the presence of both VEGFR-3 and LYVE-1, the special markers for LE cells (*Fig. 1C,D*). The results confirm that the cells isolated are indeed LE cells.

Inhibitory Effect of Angiostatin and Thalidomide on LE Cells

TABLE 1
Inhibitory Effect of Angiostatin and Thalidomide on Proliferation and Migration of LE Cells by Wound Closure Method (mean_standard error)

	control	0.5μg/ml	0.7μg/ml	1.0μg/ml
1. angiostatin				
cell number	30.3 ± 3.3	26.2 ± 3.9	23.1 ± 2.8	19.4 ± 2.3
migration distance μm	3.3 ± 0.53	2.6 ± 0.43	2.5 ± 0.36	2.1 ± 0.18
P (vs control group)		<0.01	<0.01	<0.01
2. thalidomide				
cell number	35.5 ± 3.7	28.0 ± 2.7	20.6 ± 2.1	18.4 ± 2.2
migration distance μm	3.8 ± 0.44	2.7 ± 0.36	2.3 ± 0.26	2.1 ± 0.23
P (vs control group)		<0.01	<0.01	<0.01

TABLE 2
Inhibitory Effect of Angiostatin and Thalidomide on Proliferation of LE Cells by MTT (mean ± standard error)

Group	OD value (490nm)	P*
1. angiostatin control	0.171 ± 0.0151	
0.5 μg/ml	0.147 ± 0.0101	0.022
0.7 μg/ml	0.127 ± 0.0158	<0.001
1.0 μg/ml	0.115 ± 0.0100	<0.001
1.25 μg/ml	0.107 ± 0.0093	<0.001
2. thalidomide control	0.165 ± 0.0105	
0.3 μg/ml	0.151 ± 0.0038	0.038
0.5 μg/ml	0.145 ± 0.0062	<0.01
0.7 μg/ml	0.126 ± 0.0060	<0.01
1.0 μg/ml	0.104 ± 0.0059	<0.01
1.5 μg/ml	0.080 ± 0.0062	<0.01
vs control group		

The results are summarized in *Table 1*. Note that the number of LE cells migrated out from the scrape-wound margin and the distances which they migrated were much higher in controls than both the angiostatin and thalidomide treated ones. The decrease in cell growth rate and rate of migration were dose-dependent. This finding indicates that these two drugs exerted inhibitory effects on both the growth and mobility of LE cells. The

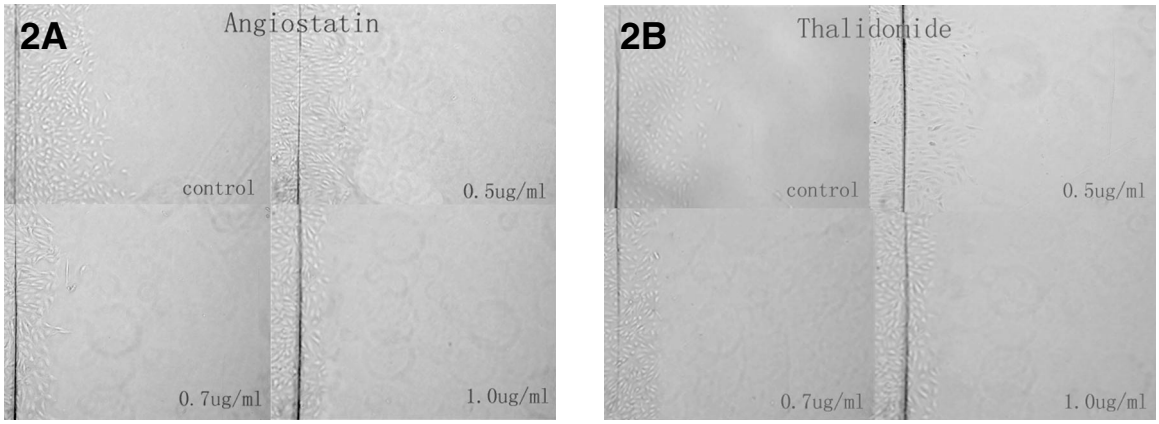

Fig. 2. Inhibition of LE cell migration by angiostatin (A) and thalidomide (B). Note that the number of LE cells that have migrated out from the scrape-wound margin and the distance they have migrated were much higher in control than in those treated with angiostatin or thalidomide. x100.

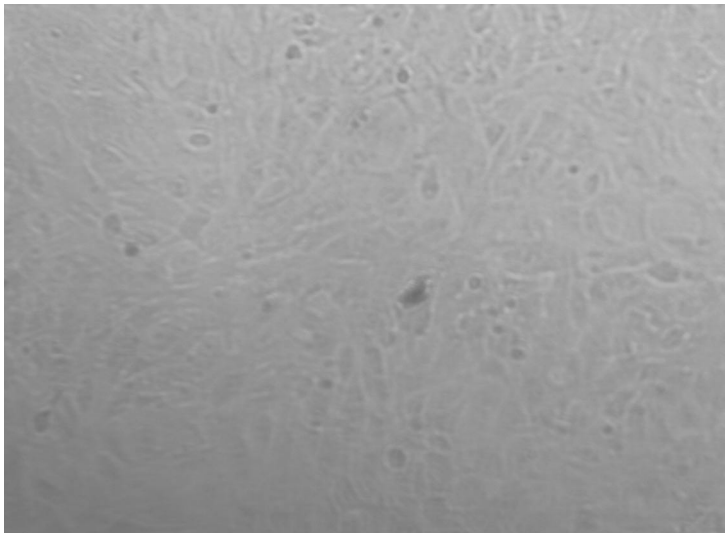

Fig. 3. High concentration of thalidomide treatment (1.5 µg/ml). Note that at this concentration, LE cells show signs of drug toxicity such as formation of granular sedimentation coupled with apoptosis. x100.

results were derived from three separate experiments.

MTT Assay

The results of MTT assays are shown in *Table 2*. Note that the results agreed well with the decreased growth rate of LE cells shown in previous section. The decrease in growth rate of LE cells here was also generally dose-dependent. However, when the concentration of angiostatin and thalidomide reached the level of 1.5 µg/ml, respectively, many LE cells showed signs of drug toxicity with substantial increase in number of detached/apoptotic cells (*Fig. 3*).

Apoptotic Assay (Hoechst Staining)

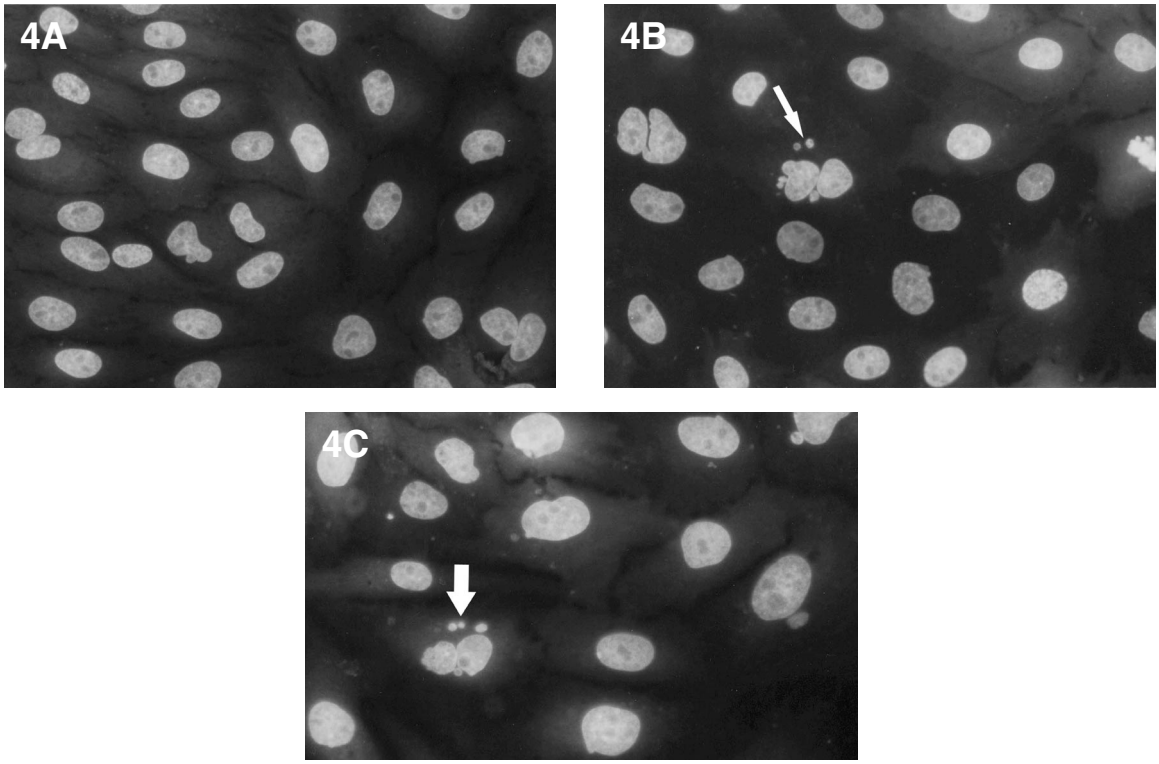

Fig. 4. Hoechst staining: Note that the nuclear profiles of LE cells in the control group (A) were larger with no apoptotic bodies found compared to treatment by angiostatin (B) and thalidomide (C). Note that after angiostatin treatment most LE cells had a smaller nuclear profile together with the appearance of apoptotic bodies (arrows). x400.

Treatment with the two drugs (i.e., angiostatin and thalidomide) induced apoptosis in LE cells. In general, the treated LE cells showed a smaller nuclear profile than the control (*Fig. 4A*) together with the appearance of apoptotic bodies (*Figs. 4B,C*).

Flow Cytometry

The results of flow cytometry assay are summarized in *Fig. 5* (A-E). In this analysis, the appearance of apoptotic cells elicited a hypodiploid nuclear spike indicating the presence of apoptotic cells. After the treatment of LE cells with angiostatin (B-C) and thalidomide (D-E), typical apoptotic spikes were observed. Note that the apoptotic spikes in the treated cases (i.e., B-C, angiostatin; D-E, thalidomide) were much more prominent than in the control (A). In the case of angiostatin, the apoptotic rate increased from 1.46% in control to 5.7% and 10.08% respectively after treatment with two different concentrations (i.e., 1.5 and 2.0 µg/ml). In the case of thalidomide (i.e., 2.0 and 3.0 µg/ml), it increased from 1.46% to 10.40% and 14.65%, respectively. *Fig. 6* summarizes the results in histogram format. The results indicated that angiostatin and thalidomide were able to promote apoptosis of LE cells in a dose-dependent manner.

DISCUSSION

Angiostatin has been shown to inhibit proliferation and migration of blood vessel endothelial cells and induce apoptosis (14). The possible mechanisms for inhibition of proliferation and migration of endothelial

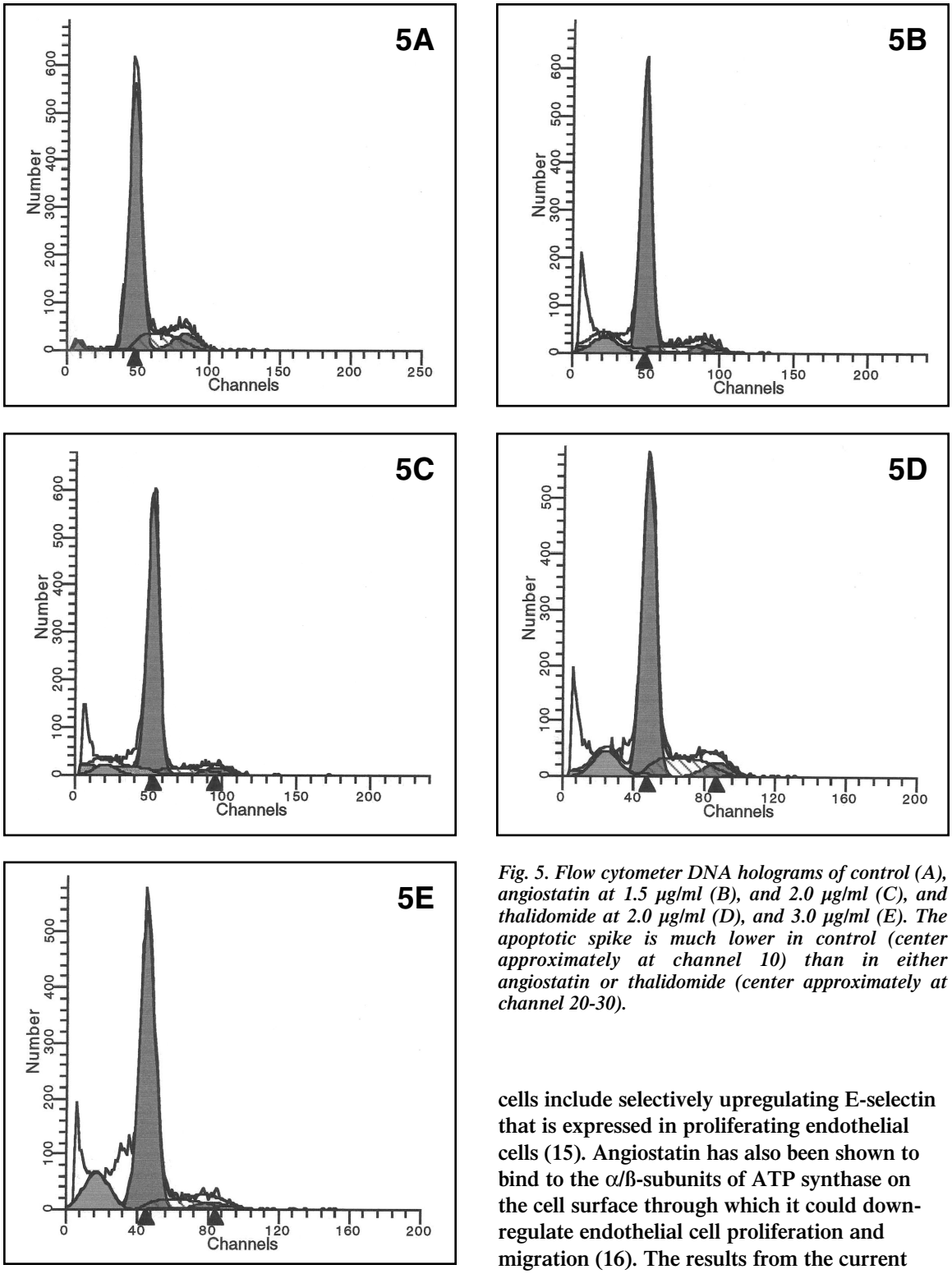

Fig. 5. Flow cytometer DNA holograms of control (A), angiostatin at 1.5 µg/ml (B), and 2.0 µg/ml (C), and thalidomide at 2.0 µg/ml (D), and 3.0 µg/ml (E). The apoptotic spike is much lower in control (center approximately at channel 10) than in either angiostatin or thalidomide (center approximately at channel 20-30).

cells include selectively upregulating E-selectin that is expressed in proliferating endothelial cells (15). Angiostatin has also been shown to bind to the α/ß-subunits of ATP synthase on the cell surface through which it could down-regulate endothelial cell proliferation and migration (16). The results from the current

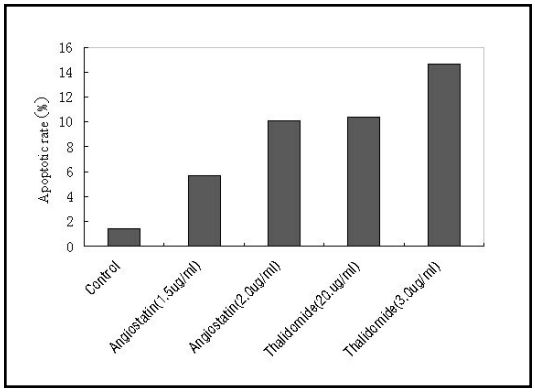

Fig. 6. Percentage of apoptotic LE cells in control, angiostatin 1.5 and 2.0 µg /ml and thalidomide 2.0 and 3.0 µg /ml treated groups

study showed that LE cells react to angiostatin treatment very similarly to blood vessel endothelial cells. As shown, angiostatin also significantly inhibited the growth and migration of LE cells and induced LE cell apoptosis in a dose-dependent manner. Whereas the mechanism involved remains unclear for LE cells, in blood vessel endothelial cells, it has been shown that activation of fas-mediated signaling pathway in involved (17). The current study has also shown that thalidomide, like angiostatin, effectively inhibits proliferation and migration of LE cells also in a dose-dependent manner and promotes apoptosis of LE cells as reflected in the significant increase in number of free floating and detached of cells. Due to the similarities in structure and function between blood vessels and lymphatics, it is not surprising that LE cells reacted very similarly to endothelial cells derived from blood vessels. It is therefore possible that the same drugs developed for antihemangiogenic purpose could also be used to inhibit growth and proliferation of LE cells, i.e., for antilymphangiogenesis.

Cancer metastasizes to distant sites through either venous or lymphatic channels or both. Grossfeld, et al (1) showed that necrosis of tumor was related not only to poor hemangiogenesis but also a lack of development of lymphatic vessels. Mattila, et al (18) showed that VEGF-C could induce lymphangiogenesis with node metastasis. Furthermore, Cao, et al (19) recently demonstrated PDGF-B induced intratumoral lymphangiogenesis and promoted lymphatic metastasis. On the other hand, Pytowski, et al (20) reported that VEGF-3 neutralizing antibody could specifically inhibit lymphatic regeneration. Shao, et al (21) recently found that endostatin and PF-4 inhibited lymphangiogenesis in a dose-dependent manner. These findings indicate that lymphangiogenesis may, like hemangiogenesis, participate actively in the growth and spread of tumors thereby justifying the development and application of drugs also inhibiting lymphangiogenesis to suppress tumor growth and retard lymphogenous spread of tumor.

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