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DIFFERENT EFFECTS OF ANGIOGENESIS INHIBITORS IFN-α AND TIMP-1 ON LYMPHANGIOGENESIS

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ABSTRACT

This study was designed to examine the effects of angiogenesis inhibitors IFN-α and TIMP-1 on lymphangiogenesis. We cultured lymphatic endothelial (LE) cells from pig thoracic ducts and performed morphological observations using light microscopy, TEM, and confocal microscopy to confirm their lymphatic origin. We tested these cells for growth inhibition by angiogenesis inhibitors IFN-α and TIMP-1 using both the scraping line and MTT methods. In addition, we analyzed apoptosis using the Hoechst and Caspase staining methods. Finally, we tested IFN-α and TIMP-1 using in vivo inhibitory assays. By morphological observations, all LE cells in vivo and in vitro were found to be of very similar morphology. Both in vitro inhibitory assays of scraping line and MTT showed significant differences for the IFN-α treatment ($p<0.01$) and no significant difference for TIMP-1. Hoechst and Caspase apoptosis assays demonstrated that IFN-α could induce apoptosis of LE cells, and TIMP-1 had little effect. IFN-α and TIMP-1 inhibitory in vivo assays showed a lack of healing following IFN-α treatment compared to control and TIMP-1 treatment. In summary, these different angiogenesis inhibitors have different effects on lymphangiogenesis. IFN-α inhibits proliferation and migration of LE cells in a dose-dependent fashion and induces apoptosis of LE cells while TIMP-1 has no significant inhibitory effects on proliferation, migration, or inducing apoptosis.

Keywords: angiogenesis inhibitors, lymphangiogenesis, endothelial cell, lymphatic; apoptosis, IFN-α, TIMP-1, metalloproteinases

In 1971, J. Folkman (1) first described the concept that growth and metastasis of the tumors depended on formation of blood vessels in the tumor. He also advanced a hypothesis that progression (and regression) of the tumor could be obtained with anti-vascular treatment. At the present time, this hypothesis is generally accepted, and the growth of solid tumors depends on the quantity of two kinds of cells, tumor cells and endothelial cells of the blood vessels in the tumor. They depend on each other and have a close relationship. If either of these cells is decreased, the other will also be decreased. Therefore, any treatment which is able to inhibit one of these cell types possesses a potential anti-tumor effect. The therapy for directly killing tumor cells has been termed anti-tumor therapy while inhibition of the vascular system is generally called anti-angiogenesis. Because anti-tumor treatment frequently has serious side effects, anti-angiogenesis therapy has become a new and hopeful target for anti-tumor effect. This kind of treatment has also been called tumor angiogenesis inhibition (TAI).

The route of metastasis taken by cancer cells after the initial break-off from its original site can be by lymphatic channels, venous passages or both (2,3). In the past three decades, extensive research has been focused on the relationship of angiogenesis and growth of tumors, and different strategies have been developed to stop the growth and spread of cancers by restricting angiogenesis. One involves 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 to death. This approach has achieved a calculated success. Through the study of angiogenesis we now have a much better understanding of the factors regulating angiogenesis in the blood vasculature. However, there has been much less attention to the factors regulating lymphangiogenesis, i.e., formation of new lymphatic vessels and underlying mechanisms. It is reported that there is lymphangiogenesis in tumors and that lymphangiogenesis has a close relationship with tumor metastasis (4). He et al (5) demonstrated that during tumor lymphangiogenesis and cancer cell dissemination via the lymphatics, the newly formed lymphatic vessels sprout from the pre-existing local lymphatic network. It would appear that to be effective in treating cancers, in addition to restricting the growth of new blood vessels in the cancers, inhibiting the formation of new lymphatic vessels may also be an important therapeutic goal. In an attempt to combine these two inhibition pathways, we designed this study to examine the potential effects of the angiogenic inhibitors IFN-α and TIMP-1 on the proliferation and migration of lymphatic endothelial cells, i.e., lymphangiogenesis. Further, we also studied the mechanisms used by these angiogenic inhibitors to influence lymphangiogenesis.

MATERIALS AND METHODS

Origin, Isolation, and Culture of Lymphatic Endothelial Cells

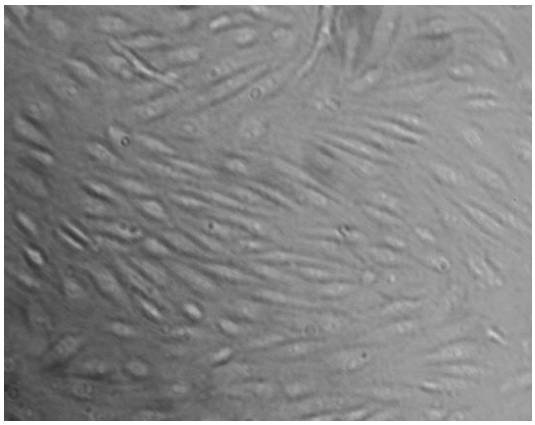

Fig. 1. 2-3 weeks after isolation and proliferation, LE cells form a confluent monolayer. Original magnification 100X.

We have previously described our isolation and culture procedures (6). Briefly, lymphatic endothelial cells were obtained from pig thoracic ducts. Fresh pig thoracic ducts were obtained at the local abattoir and kept fresh until use. A 0.1% collagenase (Sigma, Type IA) solution in PBS was used to digest LE cells and cells 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 (*Fig. 1*), and we utilized cells at second or third passage for this research.

Characterization of LE Cells

To confirm the lymphatic origin of the cells, we characterized them first by the presence of Factor VIII-related antigen (von Willebrand factor) as a specific marker for all endothelial cells, and then by VEGFR-3 and LYVE-1 as specific markers for LE cells.

For Factor VIII-related antigen staining: LE cells were grown on glass coverslips to confluency and fixed in 95% alcohol. After PBS washing, 10% sheep serum was added at room temperature (RT) for 10 min, and then antibody against Factor VIII (Sigma, 1:200.) was added and incubated at 37°C for 2 hrs. This was followed by washing in PBS and

addition of secondary antibody (sheep anti-rabbit IgG-FITC, Peking Zhongshan Bioengineering Company, 0.1 mg/ml) and incubation at 37°C for a further 10 min. The cells were then observed using a fluorescent microscope.

For VEGFR-3 (7) and LYVE-1 (8) staining: Cells grown on coverslip were fixed with 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, for 20 min in RT. After PBS washing, the coverslip was treated with 10% sheep serum at 37°C for 30 min before the addition of the primary antibody (either anti-VEGFR-3, (Sigma, 5 µg/ml, or anti-LYVE-1, AngioBio, 25 µg/ml) and incubated at 4°C overnight. Following PBS washing, the cells were treated with second antibody (sheep anti-rabbit IgG-FITC, 0.1 mg/ml) and incubated at 37°C for 1 h. The cells were examined by laser confocal microscope (Zeiss) after PBS washing.

We used TEM to further characterize the lymphatic nature of these cells. Cells at second passage were digested, centrifuged and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 for 2 hrs at RT and postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.2 for 1 hr) at 4°C and then processed for epoxy embedding, sectioning for electron microscopic observation, and examined under JEM-1200EX electron microscope. Sample preparation of in vivo LE cells from the pig thoracic duct followed the routine methods (9).

Cell Migration and Proliferation Assays (10)

Cell migration was assessed using the scrape method. 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 10 mm in the middle of the confluent LE cell culture. The cells were then allowed to grow for a further 24 hrs in medium containing different concentrations of IFN-α (Peprotech, 800 ng/ml, 1500 ng/ml, 2000 ng/ml) or different concentrations of TIMP-1 (Calbiochem, 800 ng/ml, 1500 ng/ml, 2000 ng/ml) before analysis. Controls were cultured in identical conditions without addition of the drugs. Migration rates of the cells were determined by measuring the distance from scraped line to the cells furthest from the scraped line, and we also counted cell number in migration area from LM pictures.

The growth rate of the cells was measured by MTT proliferation assay (11). LE cells (5 x 104 cells/ml) were seeded in each well into 96-well plates. IFN-α at various concentrations (1000, 2000 ng/ml) or TIMP-1 (1000 ng/ml, 2000 ng/ml) were added to the culture 24 h later. Control cultures were untreated. After 24 hr, 100 µl of 0.5 mg/ml of MTT reagent was added to each well and then incubated for 4 hrs 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 presented as the percentage of the treated cells over the untreated cells.

We also tested IFN-α and TIMP-1 in an in vivo inhibitory assay. Two rabbits (~2.5 kg) were intraperitoneally anesthetized with 20% urethane (5ml/kg). The distal dorsal paws of each posterior limb were intradermally injected with Patent blue to reveal the lymphatics (*Fig. 2*). Then the identified lymphatics of both sides were separated and cut. Following the operation, IFN-α (1 million IU/qd, tid) or TIMP-1 (3000 ng/ml/qd, tid) was injected around the wound of each left posterior limb. The two right posterior limbs were injected with 0.9% NaCl solution as the control. At day 16 post operation, the distal ends of both posterior limbs were again intradermally injected with Patent blue respectively to determine if healing had occurred by leakage of blue dye from cut (and unhealed) lymphatics.

Apoptotic Assays

For Hoechst assays, we seeded 0.5 ml of

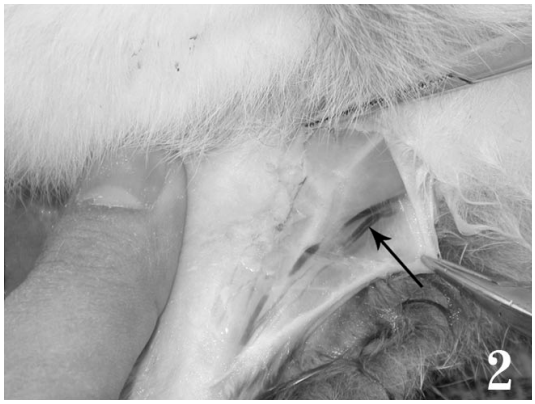

Fig. 2. Pre-excision photograph demonstrating the identified lymphatic channel (arrow) in the upper portion of the hindlimb following intradermal injection of Patent Blue dye into the dorsum of the paw. The arrow indicates the revealed lymphatic by the blue dye.

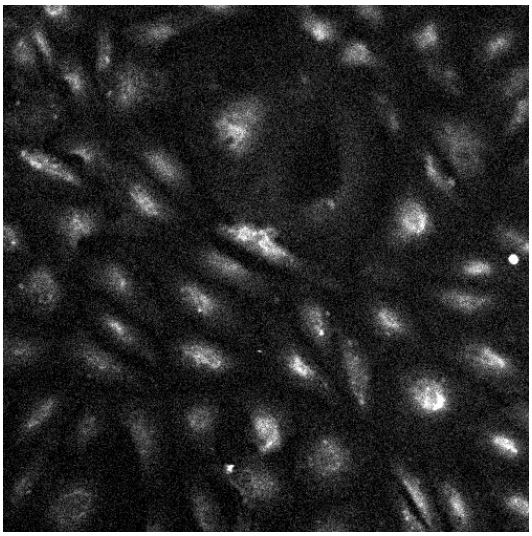

Fig. 3. Lymphatic origin of isolated cells is confirmed by immunohistochemical staining with VEGFR-3. Original magnification 20X

LE cells (5 x 104 cells/ml) onto coverslips in 24-well plates and cultured them for 24 hrs. IFN-α (control, 1500 ng/ml) or TIMP-1 (control, 1500 ng/ml) was added to the each well and cultured for a further 24 hrs. 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 (33258, Sigma, 10 μg/ml) for 10 min before examination by a fluorescent microscope.

For Caspase-3 staining (The kit was from Peking Zhongshan Bioengineering Company.), cells were seeded on coverslips in 24-well plates. IFN-α (control, 1500 ng/ml) or TIMP-1 (control, 1500 ng/ml) was added to each well with incubation for a further 24 hr. Cells were fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 for 30 min at RT. After PBS washing, coverslips were treated with 10% sheep serum at 37°C for 30 min before the addition of the first antibody (Caspase-3, 1:100) and incubated at 4°C for 1 hr. Following PBS washing, the cells were treated with second antibody (sheep anti-rabbit, 0.1mg/ml) and incubated at 37°C for 20 min. streptavidin-biotin complex was added at 37°C for 20 min, and cells were examined by a fluorescent microscope after PBS washing.

Statistical Analysis

Data were analyzed by using Sigma Stat software and Student-Newman-Keuls test to compare the significance of difference between the control and experimental groups. Results are shown as mean ± standard error, and p values below 0.05 and 0.01 were deemed as significant and highly significant, respectively.

RESULTS

Isolation and Characterization of LE Cells

Isolated LE cells began to adhere to the culture dish and formed small clumps after 6-12 hrs in culture. The cells grew quickly out from the clumps in the next several days, and by 2-3 weeks, cells had grown to near confluence. They exhibited typical cobblestone features characteristic of LE cells.

Immunofluorescent examination revealed the presence of Factor VIII-related antigen

TABLE 1
Comparison of Morphology Between LE Cells of Pig Thoracic Ducts in Vitro and in Vivo

	LE cells of pig thoracic duct in vivo	LE cells of pig thoracic duct in vitro
1. Contour of LE cells	Elliptic, spindle, oval, irregular	Elliptic, spindle, oval, irregular round, triangle
2. Protuberances of cells	+	++
3. Vesicles	+	+++
4. Mitochondria	+	++
5. Nucleus	Large, elliptic, round or irregular	Large, elliptic, round or irregular
6. Desmosomes	+	++
7. Junctions	Close junctions: linear-like, saddle-like junctions	Close junctions: Earlier developmental stage: finger-like junctions At middle, late developmental stage: linear-like, saddle-like, finger-like

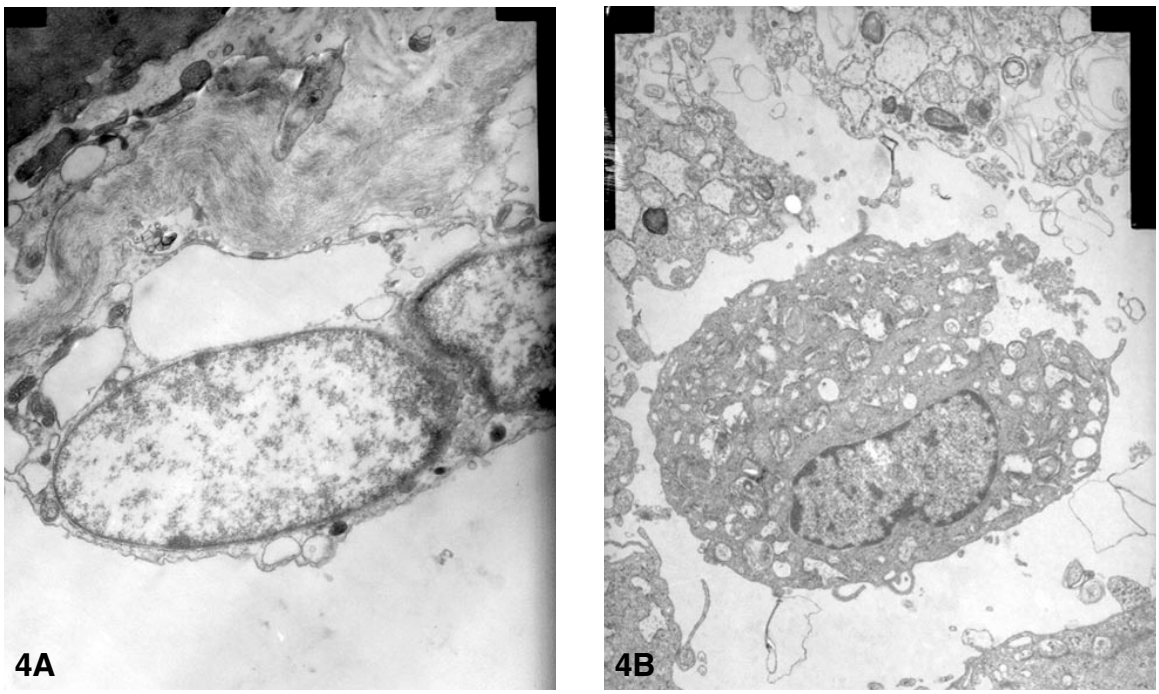

Fig. 4. TEM observations of LE cells in vivo (A) and in vitro (B). Large nucleus can be found in the center of round, oval, or irregular cells. A, 8,000X. B, 6,000X.

reactivity in the perinuclear region, which is specific to endothelial cells (data not shown). Immunohistochemical staining with both VEGFR-3 (*Fig. 3*) and LYVE-1 (data not shown) confirmed lymphatic origin of isolated cells.

Under TEM, the main ultrastructural similarities and differences of LE cells in vivo

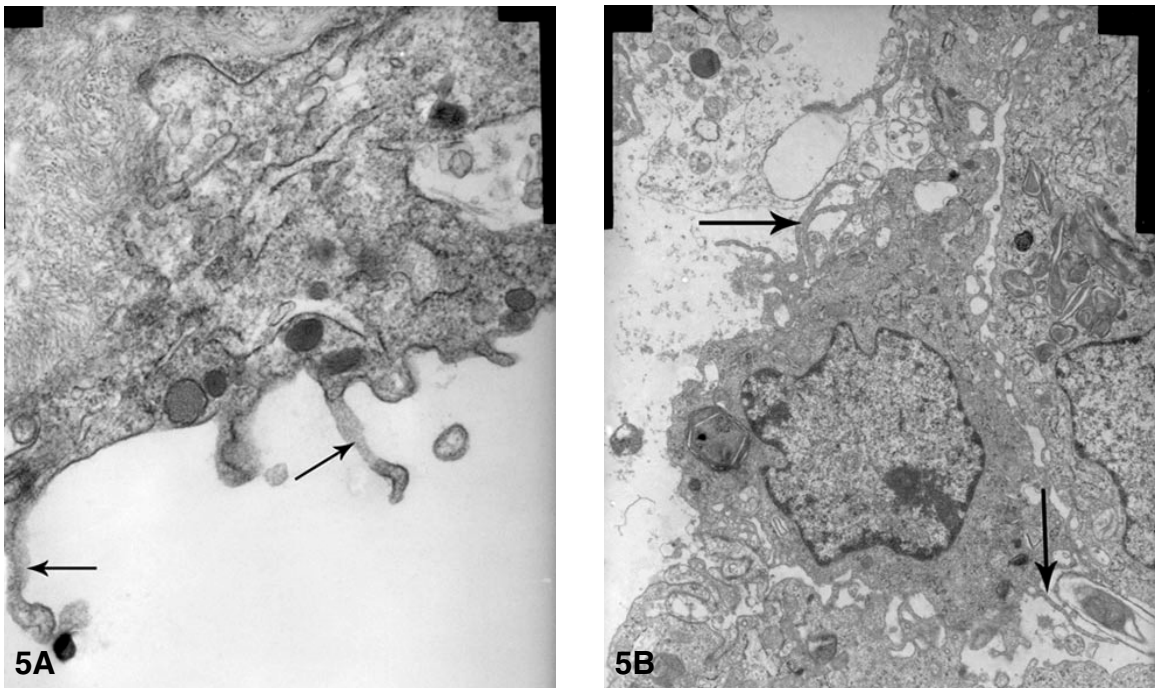

Fig. 5. TEM observations of LE cells in vivo (A) and in vitro (B) demonstration protuberances. A, 25,000X. B, 6,000X

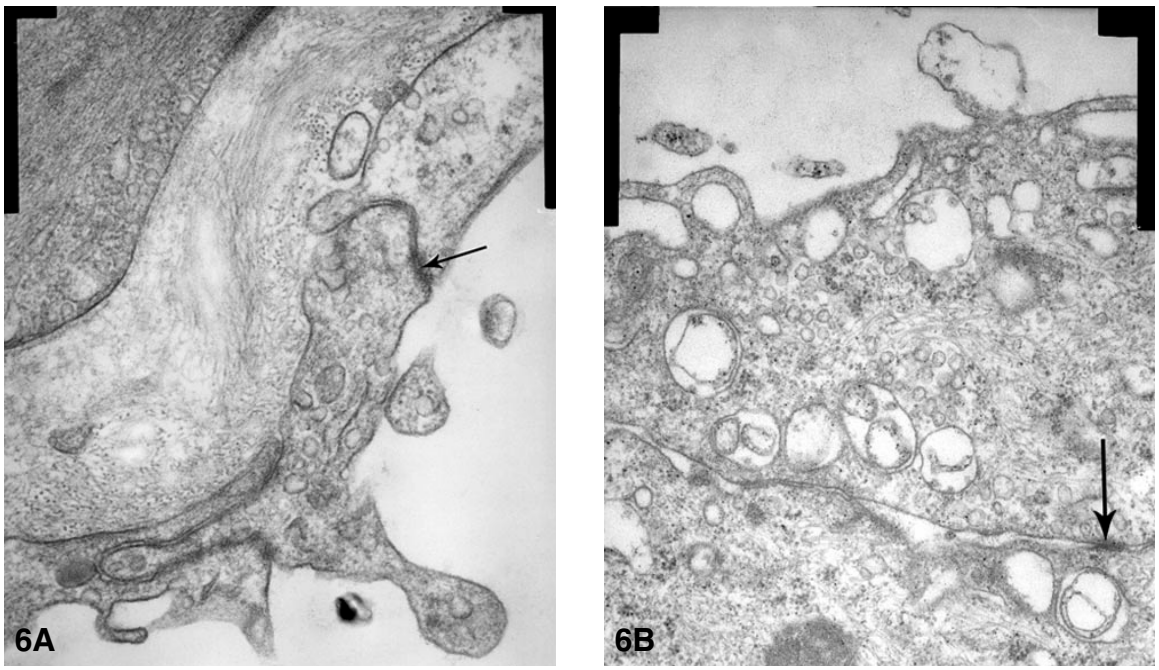

Fig. 6. TEM observations of LE cells in vivo (A) and in vitro (B) demonstrating desmosomes (arrows) located at junctions of LE cells. A, 30,000X. B, 25,000X.

TABLE 2
Inhibitive Effects of INF-α and TIMP-1 on Migration of LE Cells (Method of Artificial Wound) (Mean ± Standard Error)

Groups	Migration distance of control group	Migration distance of experimental group (concentration)	P
INF-α	4.7±1.2	1.1±0.6 (800 ng/ml)	<0.01
		0.8±0.4 (1500 ng/ml)	<0.01
		0.5±0.2 (2000 ng/ml)	<0.01
TIMP-1	3.3±0.8	3.1±0.5 (800 ng/ml)	NS
		3.2±0.4 (1500 ng/ml)	NS
		3.1±0.6 (2000 ng/ml)	NS

P=control group; NS=not significant

TABLE 3
Inhibitive Effects of INF-α and TIMP-1 on Proliferation of LE Cells (Method of Artificial Wound) (Mean ± Standard Error)

Groups	Migration distance of control group	Migration distance of experimental group (concentration)	P
INF-α	18.3±5.4	3.4±1.1 (800 ng/ml)	<0.01
		2.3±1.2 (1500 ng/ml)	<0.01
		1.7±1.5 (2000 ng/ml)	<0.01
TIMP-1	26.4±0.8	24.8±1.2 (800 ng/ml)	NS
		24.3±1.3 (1500 ng/ml)	NS
		24.7±1.0 (2000 ng/ml)	NS

P=control group; NS=not significant

and in vitro are summarized as follows and in *Table 1*: 1) The contours of most LE cells both in vitro and in vitro were elliptic, spindle, oval or irregular, and frequently a big nucleus was located in the center (*Figs. 4A, B*); 2) LE cells both in vivo and in vitro demonstrated multiple protuberances with more found in vivo than in vitro (*Figs. 5A, B*); 3) There were many vesicles seen both in vivo and in vitro with higher frequency in vitro; 4) Most junctions between LE cells were in saddle-like or linear-like forms in vivo; during early stages of development, junctions between LE cells in vitro appeared with many finger-like junctions which are formed by the protuberances. With age, they became linear-like, saddle-like, or finger-like junctions; 5) As further development of LE cells progresses in

TABLE 4
Inhibitory Effects of INF-α and TIMP-1 on Proliferation of LE Cells (MTT)

Groups	OD value (490 nm)	P
IFN-α		
Control	0.181±0.02	
1000 ng/ml	0.146±0.02	NS
2000 ng/ml	0.093±0.01	<0.01
TIMP-1		
Control	0.471±0.02	
1000 ng/ml	0.464±0.01	NS
2000 ng/ml	0.418±0.02	NS

P=control group; NS=not significant

vitro, desmosomes would be found between LE cells (*Fig. 6A, B*).

Cell Migration, Proliferation, and Inhibitory Assays

Results of the artificial wound assay for migration and proliferation are summarized in *Tables 2 and 3*. The total number of LE cells migrating from the scrape-wound margin and the distance which they migrated were much higher in control than IFN-α treated one. The decrease in cell growth rate and rate of migration were dose-dependent. This indicates that IFN-α exerted an inhibitory effect on both the growth and mobility of LE cells. The results were derived from three separate experiments. TIMP-1 treated groups were not significant.

Results of MTT assays of INF-α are shown in *Table 4*. The results agreed well with the decreased growth rate demonstrated by the wound assay (above). The decrease in growth rate of LE cells here was generally also dose-dependent. The results were derived from three separate experiments. Results from TIMP-1 treated groups were not significant.

In Vivo Inhibition Assays

Sixteen days following operation, intradermal injection of Patent blue dye demonstrated healed (no leak of dye) in the control and TIMP-1 legs and non-healed (serious leak) in the leg with IFN-α treatment (*Fig. 7*).

Apoptotic Assays

Hoechst staining demonstrated that treatment of LE with IFN-α induced apoptosis. In general, the IFN-α group displayed apoptotic bodies while none were seen in the control (*Fig. 8*). Apoptotic cells were also observed in the IFN-α treatment group by Caspase-3 staining (*Fig. 9*). TIMP-1 had little effect on inducing apoptosis of LE cells by either method (data not shown).

DISCUSSION

Interferon was first described by Isaacs and Lindenmana in 1957 (12). After many years of research, further classifications of IFN-α, IFN-α and IFN-α have been developed. In the IFN-α family, there are at least 20 members with IFN-α A and IFN-α D

Fig. 7: In vivo inhibitory assay demonstrating leakage (lack of healing) of blue dye from the root of the limb following intradermal injection into the dorsum of this IFN-α limb

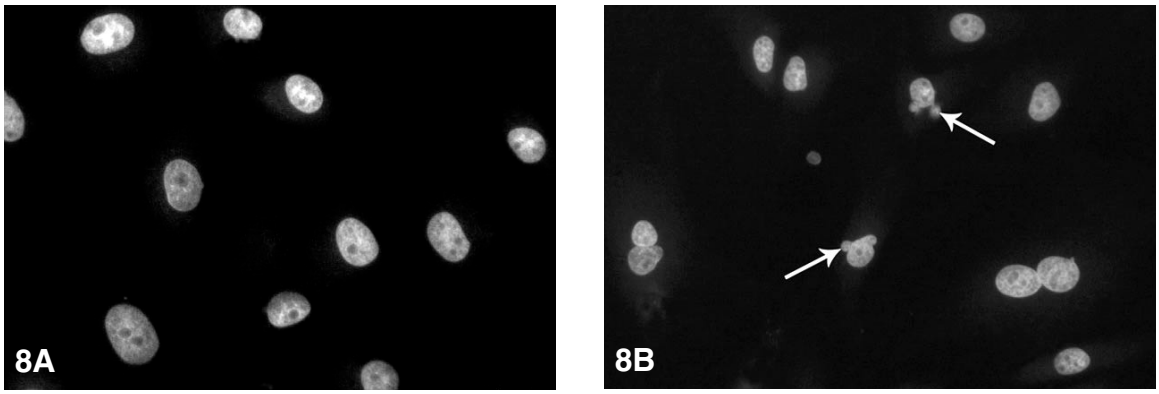

Fig. 8: Hoechst staining of LE cells in control (A) and IFN-α (1500 ng/ml) treated groups demonstrating apoptosis (arrows indicate the apoptotic bodies). A and B, 200X.

most common in humans. The main biological functions are anti-proliferation of cells, and anti-virus and immune response (13-14). Clinically, interferon has been widely applied for treatment of tumors (15). IFN-α may induce apoptosis in human dermal microvascular endothelial cells (16-17) and inhibit angiogenesis (18-19). The current study has shown that IFN-α could effectively inhibit proliferation and migration of LE cells also in a dose-dependent manner. Furthermore, it promoted apoptosis of LE cells as reflected in the significant increase in number of free floating and detached cells (data not shown).

Tissue inhibitors of metalloproteinases (TIMPS) are a family of endogenous inhibitors that regulate activation and activity of MMPs. They have been shown in animal models to be capable of the inhibition of

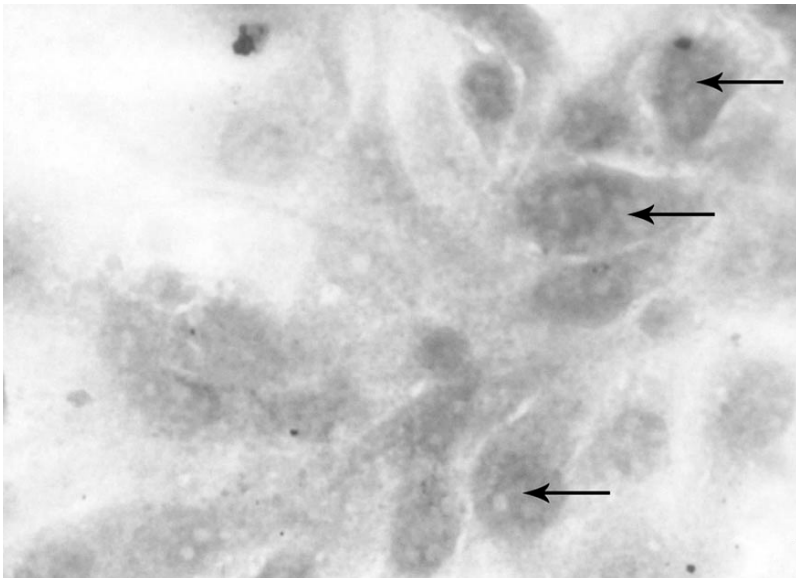

Fig. 9: Caspase-3 staining demonstrating apoptotic cells in the IFN-α experimental group. (arrows indicate apoptotic cells). 400X.

tumor cell invasion and metastasis. TIMPs may also be involved in other diseases such as arthritis and periodontal disease. TIMP-1 is a 184 amino acid glycoprotein of 28.5 kDa (20-21) which preferentially binds and inhibits MMP-1 through interaction with the catalytic domain of the MMP protein. TIMP-2 is a 194 amino acid glycoprotein of 21 kDa with 43% and 44% homology to TIMP-1 and TIMP-3 respectively. TIMP-2 inhibits the activity of all active MMPs and regulates MMP-2 (Kda-5nM). As with TIMP-1, TIMP-2 has been shown to have erythroid-potentiating activity and cell growth-promoting activity. TIMP-3 is present in the eye, binds tightly to the extracellular matrix, and has been shown to inhibit TNF-α converting enzyme (22). A mutation in TIMP-3 is found in Sorsby's fundus dystrophy, a dominantly-inherited form of blindness (22). TIMP-4 blocks the activities of several matrix metalloproteinases (MMPs) implicated in the arthritic cartilage erosion.

We have studied the influences of a series of angiogenesis inhibitors on lymphangiogenesis, including endostatin, PF-4, angiostatin, thalidomide, IFN-α and TIMP-1. The results have shown that endostatin, PF-4; angiostatin, thalidomide and IFN-α can inhibit the proliferation and migration of LE cells and were dose-dependent. The mechanism of action may be by inducing apoptosis of LE cells. According to our current research, TIMP-1 could not inhibit growth and proliferation of LE cells, and it had little effect on inducing apoptosis of LE cells. This confirms that the different angiogenesis inhibitors have different effects on lymphangiogenesis. The reasons and mechanisms are not clear and need further study.

Cancer cells metastasize to distant sites through either venous or lymphatic channels or both. In view of the importance of lymphatic vessels in spread of cancers, development and application of drugs against lymphangiogenesis, in addition to hemangiogenesis, may further improve the efficiency of inhibition of the spread of tumor through the lymphatic system. The study of inhibition of lymphangiogenesis targeted to LE cells may provide new understanding and strategies for cancer therapy.

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