Lymphology 38 (2005) 1-8

INFLUENCE OF ANGIOGENESIS INHIBITORS, ENDOSTATIN AND PF-4, ON LYMPHANGIOGENESIS

X.J. Shao, F.M. Xie

Department of Anatomy, Medical College, Qingdao University, Qingdao, PR China

ABSTRACT

This study was designed to screen potential safe and effective inhibitors of lymphangiogenesis. Lymphatic endothelial cells from pig thoracic duct were isolated and cultured. The control group, 3 endostatin, and 3 PF-4 experimental groups were tested for effects on proliferation and distance of migration of the cultured cells by two methods (method of the scraping line and MTT assay), and observations by light, confocal, and electron microscopy were also made. Total cells migrating past the scrape line for the endostatin control group was 28.6±1.2 (mean ± standard error) and the 3 endostatin experimental groups (50ng/ml,100ng/ml, and 150ng/ml), respectively, were 17.5±0.6, 10.5±0.5, and 4.8±0.3 (all p<0.05 cf control). Migration distance for the endostatin control group was 381.7±9.67μm, and the migration distance of the 3 endostatin experimental groups, respectively, were 252.9±5.58, 164.5±7.09, and 91.2±7.98μm (all p<0.05). Cell migration number for the PF-4 control group was 28.3±1.0 compared with doses of 40ng/ml, 80ng/ml, or 120ng/ml of PF-4, respectively, were 13.6±0.7, 9.5±0.6, and 4.6±0.4 (all p<0.05 cf control). Migration distance of cells in PF-4 control group was 419.9±5.87μm, and the 3 PF-4 experimental groups, respectively, were 199.2±8.16, 152.5±7.28, and 104.2±6.70μm (all p<0.05 cf control). The MTT assay confirmed that as the concentrations of endostatin and PF-4 were increased, the inhibitory effect was increased. We conclude that endostatin and PF-4 are able to inhibit the migration and proliferation of lymphatic endothelial cells, and these effects are dose-dependent.

Lymphangiogenesis, the formation of new lymphatics from pre-existing vessels, is likely a prerequisite for many physiological and pathological processes such as embryonic development, organ transplantation, wound healing, regeneration of tissue and organs, and growth and metastasis of tumors. At present, relatively little is known about the molecular mechanisms and influencing factors in lymphangiogenesis (and lymph vasculogenesis) compared to blood vessel angiogenesis (and blood vasculogenesis), but information is steadily accumulating (1). In addition, research on tumor angiogenesis has made remarkable progress (2-5), and the discovery of growth factors and inhibitors of hemangiogenesis have led to further discoveries relevant to the field of lymphangiogenesis (6). Recently, investigations have focused on growth factors such as the VEGF family (7-9) and bFGF (10) for stimulation of lymphangiogenesis and agents such as anti-VEGFR-3 for lymphangioinhibition (11). This study was designed to examine the potential influence of the hemangiogenesis inhibitors endostatin and PF-4 on lymphangiogenesis in an effort to find a safe, effective, and practical inhibitor of lymphangiogenesis, so as to limit local lymphangiogenesis and

possibly retard the spread of tumors through the lymphatic system.

MATERIALS AND METHODS

Origin of Lymphatic Endothelial Cells and Experimental Group

Lymphatic endothelial cells were obtained from the pig thoracic duct. The experimental groups were divided into endostatin groups at control, 50 ng/ml, 100 ng/ml, and 150 ng/ml, and PF-4 groups at control, 40 ng/ml, 80 ng/ml, and 120 ng/ml.

Isolation, Culture Of Lymphatic Endothelial Cells (12-18)

Fresh pig thoracic ducts were obtained at the local abattoir and kept fresh until brought to the laboratory. To visualize the thoracic duct, the lymph node in the distal end was injected with a solution of 1% Evans blue dye in phosphate buffered saline. The thoracic duct was dissected and a 10-15cm length was skeletonized from fat. The distal end was cannulated with polythene tube, and the duct was flushed with D-Hanks to remove residual dye and lymph, and a 0.1% collagenase (Sigma, Type IA) solution in PBS was injected through the tube. The thoracic duct was kept full and placed in a humid state for 10 minutes at 37°C. After incubation, the collagenase solution was collected and the duct was flushed with medium. The total cell suspension was centrifuged at 1,000 rpm for 10 minutes, the supernatant removed, and the cells suspended in medium supplemented with 20% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and seeded on the plate. They were maintained at 37°C in a humidified incubator with 95% air and 5% CO_2. The medium was changed every two or three days. When the cells reached confluence and formed a monolayer, subcultures were obtained by digestion of primary culture with 0.125% trypsin and 0.01% EDTA (Sigma).

Identification of Lymphatic Endothelial Cells

LEC were identified by VEGFR-3 (Sigma) and LYVE-1 (AngioBio) (19,20). The cells were grown on a cover glass to a confluent monolayer and fixed with 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 for 20 min at RT. After PBS washing, 10% sheep serum was added at 37°C for 30 min and the primary antibody (VEGFR-3 5µg/ml or LYVE-1 25µg/ml) was added to incubate at 4°C overnight. Following PBS washing, the second antibody (sheep anti rabbit IgG-FITC, 0.1mg/ml) was used at 37°C for 1 hour. After PBS washing, the cells were observed under laser scanning confocal microscope (Zeiss).

Inhibitory Assays

Endostatin and PF-4 were respectively obtained from Calbiochem and Peprotech. They were diluted into different concentrations in PBS (PBS used alone for control wells) and were used with the second or third generation of lymphatic endothelial cells. The medium used for all assays was DMEM (Hyclone) supplemented with 20% fetal calf serum (Gibco), 100 units/ml penicillin, 100µg/ml streptomycin, and 2 mM L-Glutamine.

Method of the scraping line

Using second passage lymphatic endothelial cells at confluence, the monolayer was damaged by removing a portion (about 10mm in width) of the cells using a scraper (rubber policeman) at the central part of the plate. The scraped cells were flushed with D-Hanks. The remaining cells were further incubated for 12 hours in the absence and presence of endostatin at 50, 100, or 150 ng/ml or PF-4 at 40, 80, or 120 ng/ml. Migration of the cells was determined by measuring the distance from scraped line to the cells furthest from the scraped line and counting the cell number in the area after photographing the monolayer.

The MTT assay (21-22)

Tetrazolium salt MTT [3-(4,5-dimethylthiazol -2yl)], 5-diphenyl tetrazolium bromide) was used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation as previously described. The lymphatic endothelial cells diluted to a concentration of 5×10^4 cells/ml and 0.1 ml were placed into each well of a 96-well plate. After 24 hours culturing, endostatin respectively in 300 ng/ml, 500 ng/ml and 700 ng/ml or PF-4 respectively in 900 ng/ml, 1,200 ng/ml, and 1,500 ng/ml, or control was added to the wells. Following an additional 24 hours of culturing, 100µl of 0.5 mg/ml MTT was added in each well. 8 wells were used for each concentration, and each concentration was repeated 8 times.

Electron Microscopic Observation

Transmission electron microscopy

Lymphatic endothelial cells at the second passage were digested, centrifuged and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 for 2 hours at RT and postfixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.2 for 1 hour) at 4°C and then processed for epox embedding and sectioning for electron microscopic observation and examined under JEM-1200EX electron microscope.

Scanning electron microscopy

Lymphatic endothelial cells at the second passage were grown to confluence on glass cover slips, fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 for 2 hours at RT and postfixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.2 for 1 hour) at 4°C, rinsed in buffer and dehydrated in an ethanol series to 100% and then infiltrated with amyl acetate and critical point dried using CO_2 in a critical point dryer. The critical point dried cover slips were coated with gold and examined using a JEOL JSM-840 scanning electron microscope.

Statistical Analysis

All data were obtained by three independent experiments and the results shown as mean ± standard error. Statistical significance was calculated using Sigma Stat software.

RESULTS

Morphological Characteristics of Lymphatic Endothelial Cells

After 24 hours in culture, in the primary passage, lymphatic endothelial cells typically formed a small clump consisting of 5-6 cells *(Fig. 1)*. After 2-3 weeks in culture, the cells grew to near confluence with most of the cells demonstrating a uniform cobblestone appearance characteristic of endothelial cells and demonstrating contact inhibition *(Fig. 2)*. Under the inverted microscope, lymphatic endothelial cells were flat, elongated or polygonal shape. The sizes of the cells were different. The nucleus was oval, and the nucleus area was more prominent than the non-nucleus area. Under SEM, confluent lymphatic endothelial cells showed a continuous sheet of cells growing in a monolayer *(Fig. 3)*. There were finger-like protuberances between the cells, and the cell surface was prominent. Under TEM, during early stage of development of lymphatic endothelial cells, there were many long protuberances from the cells *(Fig. 4)*. As lymphatic endothelial cells further developed, junctions occurred between the cells. Some structures similar to desmosomes were found *(Fig. 5)*, and the nucleus was large. The immunofluorencence studies demonstrated the presence of VEGFR-3 and LYVE-1 on the cell surface of confluent cultures *(Figs. 6,7)*.

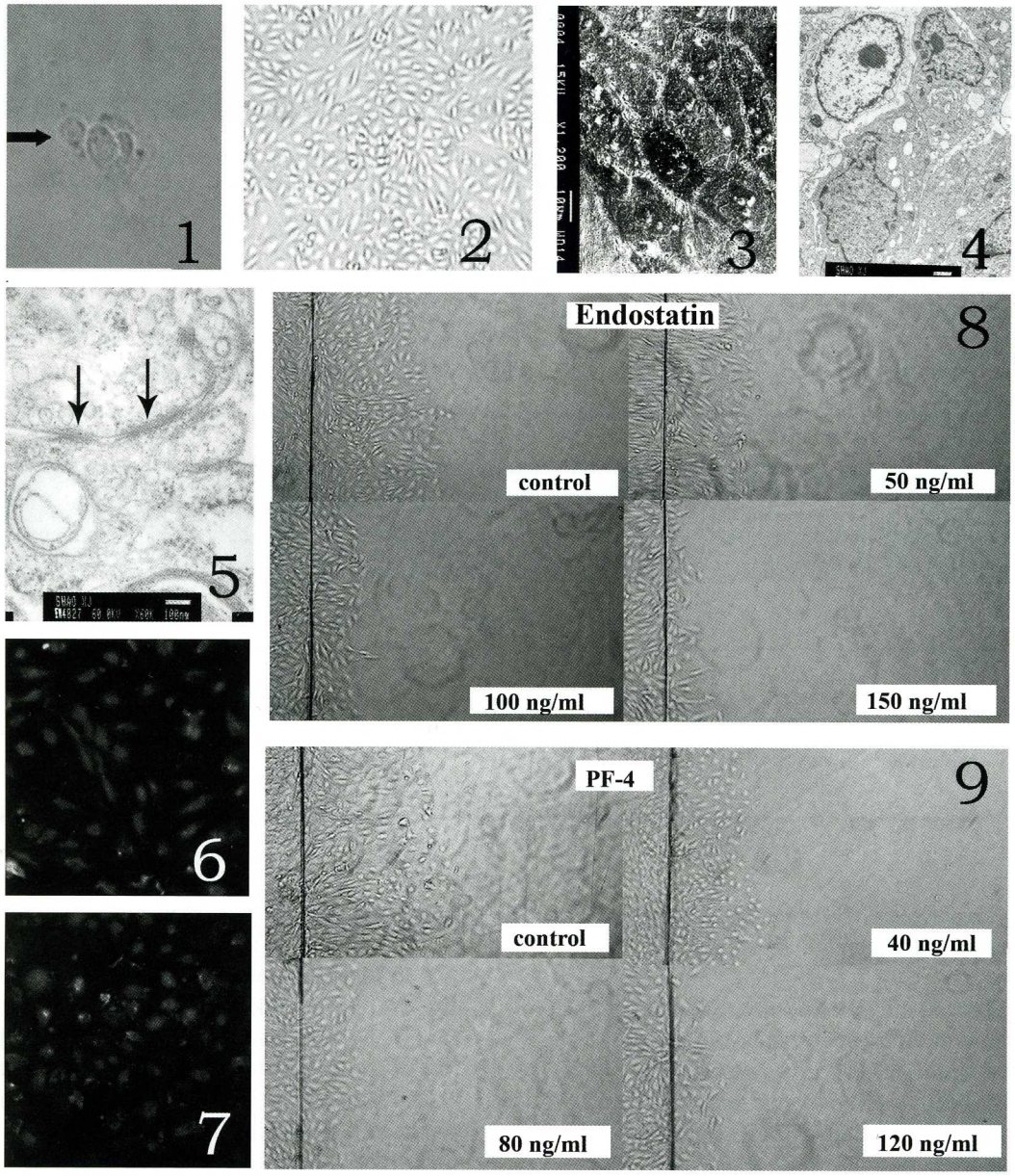

Fig. 1. Isolated clump of lymphatic endothelial cells after 12-24 hours (arrows), X100.
Fig. 2. Lymphatic endothelial cells form a confluent monolayer at 2-3 weeks, X100.
Fig. 3. SEM observation of the porcine lymphatic endothelial cells, X1200.
Fig. 4. TEM observation of the porcine lymphatic endothelial cells, X5000.
Fig. 5. TEM observation of the junction similar to the desmosome between endothelial cells (arrows), X60000
Fig. 6. The lymphatic endothelial cells following immunohistochemical staining with VEGFR-3, X200.
Fig. 7. The lymphatic endothelial cells following immunohistochemical staining with LYVE-1, X200.
Fig. 8. Migration of the lymphatic endothelial cells in control group and 50 ng/ml, 100 ng/ml, 150 ng/ml endostatin treated groups, X100.
Fig. 9: Migration of the lymphatic endothelial cells in control group and 40 ng/ml, 80 ng/ml, 120 ng/ml PF-4 treated groups, X100.

TABLE 1
Inhibitory Effect of Endostatin on Proliferation and Migration of Lymphatic Endothelial Cells (mean ± standard error)

	control	50 ng/ml	100 ng/ml	150 ng/ml
cell number	28.6±1.2	17.5±0.6	10.5±0.5	4.8±0.3
migration distance (μm)	381.7±9.66	252.9±5.58	164.5±7.09	91.2±7.98
p value		<0.05	<0.05	<0.05

TABLE 2
Inhibitory Effect of Endostatin on Lymphatic Endothelial Cells (MTT) (mean ± standard error)

	control	300 ng/ml	500 ng/ml	700 ng/ml
OD value	0.17±0.101	0.14±0.022	0.15±0.007	0.12±0.006
p value		<0.05	<0.05	<0.05

Inhibitory Effect of Agents on Lymphatic Endothelial Cells

The cell number and migration distance for the lymphatic endothelial cells which passed through the scraping line were significantly lowered by endostatin *(Table 1, Fig. 8)* and PF-4 *(Table 3, Fig. 9)*. This inhibitory action on proliferation of lymphatic endothelial cell was at a dose-dependent fashion. At 50 ng/ml endostatin or 40 ng/ml PF-4, few cells were dead while at 150 ng/ml endostatin or 120 ng/ml PF-4, many lymphatic endothelial cells became round, disconnected, floating and dead. The MTT assay also showed a dose-dependent anti-proliferative response of the lymphatic endothelial cells to endostatin *(Table 2)* and PF-4 *(Table 4)*.

DISCUSSION

Endostatin was isolated from serum of EOMA by O'Reilly and Folkman in 1997 (5). It is a 20 kD C-terminal fragment of collagen XVIII. It functions as a specific inhibitor of endothelial cell proliferation and angiogenesis, however, the mechanism is incompletely understood. Possible mechanisms include blocking signal conduction of FGF and inhibiting migration (23); down-regulating Bcl-2,Bcl-xl and promoting apoptosis of endothelial cells (24) among others. In this study, we found that it strongly inhibits proliferation and migration of LEC, in a dose-dependent manner. The mechanism(s) of action of endostatin on LECs may be the same as on endothelium of blood vessels.

PF-4 belongs to C-X-C chemokine family

TABLE 3
Inhibitory Effect of PF-4 on Proliferation and Migration of Lymphatic Endothelial Cells (mean ± standard error)

	control	40 ng/ml	80 ng/ml	120 ng/ml
cell number	28.3±0.99	13.6±0.71	9.5±0.57	4.6±0.40
migration distance (μm)	4.4±0.06	2.1±0.09	1.6±0.08	1.1±0.07
p value		<0.05	<0.05	<0.05

TABLE 4
Inhibitory Effect of PF-4 on Lymphatic Endothelial Cells (MTT) (mean ± standard error)

	control	900 ng/ml	1200 ng/ml	1500 ng/ml
OD value	0.17±0.015	0.10±0.006	0.09±0.012	0.07±0.022
p value		<0.05	<0.05	<0.05

(25).This family also includes interleukin-8 (IL-8), β-thromboglobulin, neutrophil-activating protein, interferon-inducing protein10 (IP-10), and melanocyte growth-stimulating activity. PF-4 is a 7.8-kD protein of 70 amino acid length (26). It shares homologies in particular with β-thromboglobulin and IL-8 of 51% and 31%, respectively. The crystal structure of human PF-4 has been solved to a resolution of 2.4Å by molecular replacement (27).The N-terminal residues form antiparallel β-sheet like structures. A encircles the PF-4 molecule presenting multiple potential sites for heparin binding.

PF-4 exhibits biological activity for several cell types including megakaryocytes, leukocytes, lymphocytes, and endothelial cells. It has been shown that PF-4 inhibits endothelial cell proliferation, migration, and angiogenesis *in vitro* and *in vivo* (28-29). In addition, PF-4 reduces tumor growth in vivo (30,31). Intralesional injection in mice of recombinant human PF-4 inhibited melanoma cell or HCT 116 colon carcinoma cell growth by an angiogenesis-dependent mechanism. Furthermore, virally transduced rat glioma cells with a secretable PF-4 cDNA grew slowly *in vivo* and only formed hypovascular tumors. This indicates that the vasculature is the prime target for PF-4. In addition, PF-4 is targeted *in vivo* to endothelial cells that undergo active angiogenesis (32). PF-4 may also be important as a physiological regulator of FGF activity. Indeed, platelets release during activation an inhibitor of FGF-2 activity (33), and it has been shown that this inhibitor is identical to

PF-4. Thus, PF-4 may counteract excessive angiogenic factor activity at sites of platelet activation.

The mechanism of PF-4 action is incompletely understood. Sato et al (34) have reported that PF-4 inhibits binding of FGF-2 to low-affinity proteoglycans. In agreement with the latter observation, Luster et al (35) showed that the IP-10 chemokine is associated with cell surface proteoglycans and that IP-10 binding can be composed by PF-4. In addition, a common heparan sulfate binding site may also be shared with histidine-rich glycoprotein (HRGP) as this molecule, like PF-4, displaces FGF-2 from the extracellular matrix (36). Furthermore, Gengrinowith et al (37) reported that VEGF binding to endothelial cell VEGF receptors was inhibited by PF-4. Gupta (29) showed that PF-4 intervenes at a specific point in the cell cycle by blocking the progression of endothelial cells in S-phase.

In this study, we found that PF-4 reduced the cell number and the migration distance of experimental groups in a dose-dependent manner. Due to the similarities (albeit with substantial differences) of structure and function between blood vessels and lymphatics, a similar mechanism of action for the anti-proliferation effects may nonetheless be operating.

These in vitro experiments on endostatin and PF-4 demonstrate dose-dependant anti-proliferative effects on porcine LECs and should form the basis for well-designed studies to further explore possible mechanisms and efficacy in in vivo models.

REFERENCES

1. Yancopoulos GD, S Davis, NW Gale, et al: Vascular-specific growth factors and blood vessel formation. Nature 407 (2000), 242-248.
2. Paavonen, K, P Poulakkainen, L Jussila, et al: Vascular endothelial growth factor receptor in lymphangiogenesis in wound healing. Am. J. Pathol. 156 (2000), 1499-1504.
3. Gately, S, P Twardowski, MS Stack, et al: Human prostate carcinoma cells express enzymatic activity that covers human plasminogen to the angiogenesis inhibitor angiostatin. Cancer Res. 56 (1996), 4887-4890.
4. O'Reilly, MS, L Holmgren, Y, et al: Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79 (1994), 315-319.
5. O'Reilly, MS, T Boehm, Y Shing, et al: Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. Cell 88 (1997), 277-282.
6. Kim H, DJ Dumont: Molecular mechanisms in lymphangiogenesis: Model systems and implications in human disease. Clin. Genet. 64 (2003), 282-292.
7. Jeltsch, M, A Kaipainen, V Joukov, et al: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice Science. 276 (1997), 1423-1425.
8. Matsumoto, T, L Claesson-Welsh: VEGF receptor signal transduction. Sci-STKE 112 (2001), RE21. Review.
9. Mattila Mirjami, MT, K Ruohola Johanna: VEGF-C induced lymphangiogenesis is associated with node metastasis in orthotopic MCCF-7 tumors. Int. Cancer 98 (2002), 46 -51.
10. Liu, NF, QL He: The regulatory effects of cytokines on lymphatic angiogenesis. Lymphology 30 (1997), 3-12.
11. Pytowski B, J Goldman, K Persaud, et al: Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. J. Natl. Cancer Inst. 97 (2005), 14-21.
12. Johnston, MG, MA Walker: Lymphatic endothelial and smooth- muscle cells in tissue culture. In Vitro 207 (1984), 566-572.
13. Gnepp, DR, W Chandler: Tissue culture of human and canine thoracic duct endothelium. In Vitro Cell Dev. Biol. 21 (1985), 200-206.
14. Joes, BE, LCJ Yong: Culture and characterization of bovine mesenteric lymphatic endothelium. In Vitro Cell Dev. Biol. 23 (1987), 698-706.
15. Djoneidi, M, P Brodt: Isolation and characterization of rat lymphatic endothelial cells. Microcirc. Endothelium Lymphatics 7 (1991), 61-82.
16. Leak, LV, M Jones: Lymphatic endothelium isolation, characterization and long term culture. Anat. Rec. 236 (1993), 641-652.
17. Tan, Y-Z, H Wang: Effects of extracellular matrix on lymphangiogenesis in vitro. Acta Anat. Sinica 331 (2002), 69-73.
18. Lu, W, X Shao: Progress of new techniques of lymphatic research. Acta Acdamiae Medicinae Qingdao Universitis 381 (2002), 81-82.

19. Iljin, K, MJ Karkkainen, EC Lawrece, et al: VEGFR3 gene structure, regulatory region and sequence polymophisms. FASEB 15 (2001), 1028-1036.
20. Banerji, S, J Ni, SX Wang, et al: LYVE-1, a new homologue of the CD44 glucoprotein, is a lymph-specific receptor for hyaluronan. J. Cell Biol. 144 (1999), 789-801.
21. Mosmann, T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65 (1983), 55-63.
22. Gerlier, D, N Thomasset: Use of MTT Colorimetric Assay to measure cell activition. J. Immunol. Methods 94 (1986), 57-63.
23. Hohenester, E, T Sasaki, BR Olsen, et al: Crystal structure of the angiogenesis inhibitor endostatin at 1.5A resolution. EMBO J. 17 (1998), 1656-1664.
24. Dhanabal, M, R Ramchandran, MJ Wateman, et al: Endostatin induces endothelial cell apotosis. J. Biol. Chem. 274 (1999), 11721-11726.
25. Perollet C, ZC Han, C Savona, et al: Platelet factor 4 modulates fibroblast growth factor2 (FGF2) activity and inhibits FGF2 dimerrization. Blood 91 (1998), 3289-3299.
26. Deuel, TF, PF Keim, M Farmer, et al: Amino acid sequence of human platelet factor 4. Proc. Natl. Acad. Sci. USA 74 (1977), 2256.
27. Han, ZC, L Sensebe, JF Abgral, et al: Platelet factor 4 inhibits human megakaryo-cytopoiesis in vitro. Blood 75 (1990), 1234.
28. Maione, TE, GS Gray, J Petro, et al: Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science. 247 (1990), 77.
29. Gupta, SK, JP Singh: Inhibition of endothelial cell proliferation by platelet factor-4 involves a unique action on S phase progression. J. Cell Biol. 127 (1994), 1121.
30. Sharpe, RJ, HR Byers, CF Scott, et al: Growth inhibition of murine melanoma and human colon carcinoma by recombinant human platelet factor-4. J. Natl. Cancer Inst. 82 (1990), 848.
31. Kolber, DL, TL Knisely, TE Maione: Inhibition of development of murine malanoma lung metastases by systemic administration of recombinant human platelet factor-4. J. Natl. Cancer Inst. 87 (1995), 304.
32. Hansell, P, TE Maione, P Borgstrom: Selective binding of human platelet factor-4 to regions of active angiogenesis in vivo. Am. J. Physiol. 269 (1995), 829.
33. Watson, JB, SB Getzler, DF Mosher: Platelet factor-4 modulates the mitogenic activity of basic fibroblast growth factor. J. Clin. Invest. 94 (1994), 261.
34. Sato, Y, M Abe, R Takaki: Platelet factor-4 blocks the binding of basic fibroblast growth factor to the receptor and inhibits the spontaneous migration of vasculae endothelial cells. Biochem Biophys. Res. Commun. 172 (1990), 595.
35. Luster, AD, SM Greenberg, P Leder: The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor-4 and inhibits endothelial cell proliferation. J. Exp. Med. 182 (1997), 219.
36. Brown, KJ, CR Parish: Histodine-rich glycoprotein and platelet factor-4 mask heparan sulfate proteoglycans recognized by acidic and basic fibroblast growth factor. Biochem. 33 (1994), 13918.
37. Gengrinowith, S, SM Greenberg, T Cohen, et al: Platelet factor-4 inhibits the mitogenic activity of VEGF 121 and VEGF 165 using several concurrent mechanism. J. Biol. Chem. 270 (1995), 15059.

Xujian J. Shao, MD
Department of Anatomy
Medical College
Qingdao University
Qingdao, PR China 266021