Basic Fibroblast Growth Factor-Mediated Lymphangiogenesis of Lymphatic Endothelial Cells Isolated from Dog Thoracic Ducts: Effects of Heparin

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Abstract: We have attempted to evaluate whether, similar to the angiogenesis of blood vessels, cultures of lymphatic endothelial cells (LEC) isolated from dog thoracic ducts have an ability to induce lymphangiogenesis in response to basic fibroblast growth factor (bFGF), then to examine the effects of heparin on the bFGF-mediated morphogenesis. The effects of bFGF and/or heparin on the proliferation and migration of the LEC were evaluated by changing the number of the subconfluent cells and by wound migration assay, respectively. The effects of the agents on invasion and tube formation of the LEC into a three-dimensional collagen gel and on collagen gel induced tube formation of the LEC were also investigated by a phase-contrast microscope and an electron microscope. The bFGF (10 ng/ml) caused a significant induction of proliferation and migration of the LEC, the induction of which was augmented dose-dependently by an additional treatment with heparin ranging from 1 to 100 µg/ml. The bFGF produced invasion and tube formation of the LEC into a three-dimensional collagen gel. The bFGF also facilitated to form capillary-like tubes of the LEC between two layers of collagen gels. Heparin (10 µg/ml) accelerated both processes of bFGF-mediated lymphangiogenesis of the LEC. These findings suggest that the cultured LEC isolated from dog thoracic ducts have an ability to form lymphatic capillary-like tubes in response to bFGF and that heparin accelerates dose-dependently the process of the bFGF-mediated neovascularization of lymph vessels. [Japanese Journal of Physiology, 48, 133–141, 1998]

Key words: lymphatic endothelial cells, bFGF, heparin, wound assay, tube formation.

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lymphatic endothelial cells isolated from dog thoracic ducts.

The bFGF is known to be a multifunctional pleiotrophic polypeptide [10, 11] and an angiogenetic factor [12, 13]. The importance of heparin-like substances in the bFGF signaling has been also emphasized, but the controversy over the extent of the involvement of heparin in the bFGF signaling awaits resolution [14–17].

Thus in the present study, we have attempted to examine whether the cultured LEC isolated from dog thoracic ducts, similar to bovine mesenteric lymph vessels, have an ability to induce in vitro neovascularization of lymph vessels in response to bFGF. The second goal of this study was to test the effects of heparin on the bFGF-induced morphogenesis of the lymphatic endothelial cells.

MATERIALS AND METHODS

Isolation and culture of lymphatic endothelial cells. Adult mongrel dogs of both sexes (6.0–12.0 kg body wt.) were anesthetized with pentobarbitonal sodium (25 mg/kg, i.v.) and exsanguinated via the femoral arteries. The experimental protocol was approved by the Animal Ethics Committee, Shinshu University School of Medicine, in accordance with the principles and guidelines of the American Council on Animal Care. Lymphatic endothelial cells (LEC) were isolated from dog thoracic ducts by using a modification of the methods described by Grepp and Chandler [18]. Briefly, 10 to 15 cm of the thoracic ducts were isolated and placed in cold (4°C) Hanks buffer salt solution (HBSS, Sankyo Junyaku Co., Japan). The duct was rapidly cleansed of surrounding connective tissues, and all its branches were then ligated by using sterile silk threads. The peripheral end of the thoracic duct was cannulated with a sterile polyethylene tube. Following the cannulation, each lymph vessel was flushed with cold (4°C) HBSS, then filled with a 37°C solution of collagenase (250 U/ml in HBSS) for 10 min. The collagenase solution was gently drained into a centrifuge tube, and the lumen of the duct was then washed with Eagle's minimal essential medium (MEM, Sankyo Junyaku Co.) containing 10% fetal bovine serum (FBS, Nalgene, Australia). The washing medium was also gathered into the same centrifuge tube. The tube containing the collagenase solution and the washing medium was centrifuged at 200 g for 10 min. The pellet obtained from the procedure was suspended in MEM medium containing 10% FBS, 15 mM HEPES, L-glutamine (584 mg/l), glucose (3.5 g/l), pyruvate (110 mg/l), penicillin (100 U/ml), and streptomycin (100 μg/ml) (LEC complete medium). The isolated lymphatic cells were plated into 35-mm culture dishes (Falcon Plastics, USA) that were coated with 1% gelatin. The cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air.

For subculture, the confluent LEC were treated with 0.25% trypsin–0.02% EDTA until the cells began to retract and round up. The trypsin-EDTA solution was removed and replaced with the LEC complete medium. The cells dislodged from the plastic surface were centrifuged at 200×g for 10 min. The pellet of cells was roughly resuspended into a 35-mm culture dish coated with 1% gelatin at a ratio of 1:3 (3×10^5 cells/ml), then placed in the incubator for 30 min. After this short incubation, the floating cells were removed by pipetting off the culture medium and briefly rinsing the dishes with PBS. Adherent cells were re-fed with the LEC complete medium and returned to the incubator. The LEC were used at the third or fourth passage for the present experiments.

The clones originating from the single cell were characterized by indirect immunofluorescence stained with a rabbit antihuman Factor VIII related antigens antiserum (Nordic Immunological Lab., The Netherlands) and by uptake of 1,1-diocadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate labeled acetylated low-density lipoprotein (DiI-Ac-LDL, Biomedical Tech. Inc., USA).

Silver nitrate staining of the cultured cells. The cells grown to confluence were stained with silver nitrate by using the method of Poole et al. [19] with minor modifications. The cells were pretreated with 5% glucose for 1–2 min, stained with 0.4% AgNO_3 for 1 min, rinsed with 5% glucose for 1 min, then treated with 1% NH_4Br and 3% cobalt bromide for 3 min and rinsed with 5% glucose. They were fixed in 3% formalin in PBS for 20 min, rinsed in PBS, and stained in Diff-Quick (International Reagents Corp., Japan). The slides were examined under an Olympus inverted microscope and photographed.

Proliferation of LEC. The LEC were seeded at a density of 3.0×10^5 cells/35-mm culture dish and cultured by using the MEM complete medium containing 10% FBS. After 1 d of incubation, the medium was exchanged to serum-free MEM in the absence or presence of 10 ng/ml bFGF, 10 μg/ml heparin, and 10 ng/ml bFGF+10 μg/ml heparin. After 24 h, the number of cells in each dish was counted by using a hemocytometer. Eight samples were counted and the mean values then determined.

Wound migration assay. The wound migration assay was performed according to the method of
Sato and Rifkin [20]. The confluent LEC on a 35-mm culture dish were scraped with a razor blade, washed twice with PBS, then incubated with serum-free MEM in the absence or presence of 10 ng/ml bFGF, 10 μg/ml heparin, and 10 ng/ml bFGF + 10 μg/ml heparin. After 24 h, the cells were fixed with methanol and stained with Giemsa. The numbers of cell nuclei and the maximum distance of migration crossing the starting line marked on the dish were measured in 300×600 μm square. Four different parts of each dish were counted and the mean values determined.

**Protocols of invasion and tube formation.**

Gels of type I collagen fibers solubilized from adult rat tail tendons (Sigma, USA) were prepared according to a modification of the method originally described by Montesano et al. [21]. This was achieved by quickly mixing 7 vol of the cold collagen solution (2.0 mg/ml) with 1 vol of 10×MEM and 2 vol of sodium bicarbonate (11.76 mg/ml) in a sterile dish kept on ice to prevent immediate gelatin. The cold mixture was then dispensed into 35-mm plastic culture dishes and allowed to gel at 37°C for 10 min.

For an assay in which invasion was induced from the surface of three-dimensional collagen gels, the LEC were seeded onto 0.8 ml preformed gels in 35-mm tissue culture dishes at 3.0×10⁵ cells/dish in 1.0 ml serum-free medium. The medium was changed every 2 d, and treatment with agents began when the cells reached confluence. The agents, 10 ng/ml bFGF and 10 μg/ml heparin, were added either in combination or alone into the serum-free medium, and invasion of the LEC into the three-dimensional collagen gel was examined and photographed at 48 h after the treatment with the agents under a Nikon phase contrast microscope.

In another in vitro assay for angiogenesis, the LEC were seeded into the collagen gel–coated dishes and allowed to culture for 1 d in the MEM complete medium containing 10% FBS to obtain a subconfluent monolayer on the surface of the gels. The culture medium was removed and the LEC then incubated for 2 h in serum-free MEM containing four kinds of test solution, i.e., no agent (control), 10 μg/ml heparin, 10 ng/ml bFGF, and 10 μg/ml heparin + 10 ng/ml bFGF. After the 2-h incubation, the MEM was removed, and 0.8 ml of the collagen gel mixture described above was poured on top of the first gel and allowed to polymerize at 37°C for 10 min. Fresh MEM medium containing 3% FBS was added after the collagen had gelled, and this was kept at 37°C for 24 h. The reorganization of the subconfluent monolayer of the LEC was monitored and photographed with a Nikon phase contrast microscope.

The collagen gel–induced tube formation (angiogenesis) of the LEC was identified by the intraluminal space inside the tube and quantified by using an image analyzer (Luzex III, NIREKO, Japan). The results were expressed as the length (mm) or area of tube formation (mm²) per microscopic field of view (using 10 objectives). At least four fields of view were examined for each dish of four samples.

**Observation of tube formation by using electron microscopy.** To examine collagen gel–induced tube formation of the LEC, the cultures were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. After the fixation, the collagen disks were gently removed from the dishes and trimmed into about 2×2 mm squares. The dissected samples were postfixed in 1.0% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, dehydrated in graded ethanol, and processed for Quetsol 812 embedding. Semithin or ultrathin sections were obtained with an LKB ultratome, double stained with uranyl acetate and lead citrate, and examined by using a Hitachi transmission electron microscope (JEM 1200 EX).

**Drugs.** The following agents were used: Heparin sodium, which was of an unfractionated, high molecular weight; anticoagulant pig mucosal heparin (typical activity, 170 USP units per mg.); and human recombinant bFGF expressed in E. coli. They were purchased from Sigma Chemical Co. (USA). The doses of the drugs were expressed at final concentrations in the culture dishes in the text, figures, and tables.

**Statistical analyses.** Each experimental group consisted of at least four lymph vessels taken from different dogs. Experimental values were expressed as means±SEM or means±SD. Statistical analyses were made by using either Dunnett’s test after a one-way analysis of variance (ANOVA) or Student’s t-test for unpaired observations. The differences were considered to be statistically significant when p<0.05.

**RESULTS**

**Culture of LEC.**

In the third or fourth passage, small colonies proliferated and spread to form a continuous sheet of cells. As the cultures grew to near confluence, most cells demonstrated a uniform cobblestone appearance. The also resumed the typical polygonal shape, which indicated a cessation of growth.

Immunofluorescence studies demonstrated that Factor VIII related antigens were present in the perinuclear region of all cells. Almost all the confluent cells also took up Dil-Ac-LDL with a perinuclear distribution.
Silver nitrate staining

To confirm their endothelial nature as continuous sheets of flattened cells, we utilized the silver nitrate method to outline the intercellular borders of confluent cultures. The precipitates from the silver nitrate reaction were localized along the intercellular borders of adjacent cells (Fig. 1). By staining the cells with Diff-Quick, the nuclei of the confluent cells were brightly stained and appeared as oval structures containing several prominent nucleoli.

Effects of bFGF and/or heparin on proliferation of LEC

Figure 2 summarizes the effects of bFGF on proliferation of the LEC at the third or fourth passage in the absence or presence of bFGF, heparin, and bFGF + heparin. In the control, after 24 h of culture in MEM supplemented by 10% FBS, 1 d of culture in serum-free MEM caused an increase in the number of subconfluent cells, about 2 times the number (3.0 × 10⁵

Fig. 1. A microphotograph showing the close apposition of adjacent cultured cells by using the silver nitrate method. The marker is 50 μm.

Fig. 2. Effects of 10 ng/ml bFGF and/or 10 μg/ml heparin on the numbers of subconfluent cultures of the LEC isolated from dog thoracic ducts. * p<0.01 vs. the control, † p<0.05 vs. the bFGF group.

Fig. 3. Representative microphotographs of the wound migration assay with confluent monolayers of the LEC isolated from dog thoracic ducts. The triangle shows the edge of the wound. The marker is 100 μm. The A, B, C, and D denote control (no agent), 10 μg/ml heparin, 10 ng/ml bFGF, and 10 ng/ml bFGF + 10 μg/ml heparin, respectively.

Table 1. Effects of 10 ng/ml bFGF and/or heparin at three concentrations (1, 10, and 100 μg/ml) on migration of the cultures of LEC isolated from dog thoracic ducts.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of migrated cells</th>
<th>Distance of migrated cells (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11 ± 4</td>
<td>240 ± 71</td>
</tr>
<tr>
<td>10 μg/ml bFGF</td>
<td>18 ± 4</td>
<td>257 ± 50</td>
</tr>
<tr>
<td>bFGF</td>
<td>32 ± 11*</td>
<td>398 ± 41*</td>
</tr>
<tr>
<td>+ 1 μg/ml heparin</td>
<td>34 ± 7*</td>
<td>421 ± 58*</td>
</tr>
<tr>
<td>bFGF + 10 μg/ml</td>
<td>42 ± 10* *</td>
<td>513 ± 79* *</td>
</tr>
<tr>
<td>heparin 100 μg/ml</td>
<td>43 ± 12* *</td>
<td>518 ± 62* *</td>
</tr>
</tbody>
</table>

The values are mean ± standard deviation (n=16). * p<0.01 vs. the control; * * p<0.01 vs. the bFGF group.

cells/dish) before the culture. Pretreatment with 10 μg/ml heparin also produced a similar increase in the number of cells to the number observed in the control. On the other hand, 10 ng/ml bFGF caused a significant increase in the number of subconfluent cells to about 106 cells/dish, and the bFGF-mediated induction of proliferation was then augmented significantly by an additional treatment with 10 μg/ml heparin.

**Effects of bFGF and/or heparin on migration of LEC**

The effects of bFGF and/or heparin on migration of the LEC were examined in wound migration assay. Confluent monolayers of the LEC were wounded with a razor blade and further incubated in serum-free MEM containing 10 ng/ml bFGF, 10 μg/ml heparin, or 10 ng/ml bFGF + 10 μg/ml heparin for 24 h at 37°C. The LEC that migrated from the edge of the wound were fixed and stained (Fig. 3). Migration of the LEC was facilitated by treatment with 10 ng/ml bFGF, the facilitation of which was significantly potentiated by an additional treatment with 10 μg/ml heparin (Table 1). The potentiated effect of heparin was observed dose-dependently. The summarized data are shown in Table 1.

**Effects of bFGF and/or heparin on invasion and tube formation of LEC**

To identify bFGF's capability of inducing invasion and tube formation of the LEC, a confluent LEC monolayer was formed on the surface of a three-dimensional collagen gel (Fig. 4A). Under the control condition, the LEC retained the confluent monolayer on the surface of a collagen gel. At 48 h after an addition of 10 μg/ml bFGF, the LEC invaded the underlying collagen matrix to form a network of branching cellular cords beneath the surface monolayer, as viewed under a phase-contrast microscope (Fig. 4A). A translucent intraluminal space was observed in the cellular cord, which suggested tube formation of the LEC. Heparin (10 μg/ml) facilitated the bFGF-mediated invasion and tube formation of LEC (Table 2).

**Effects of bFGF and/or heparin on collagen gel–induced lymphangiogenesis of LEC**

In another **in vitro** neovascularization assay, a subconfluent monolayer of the LEC established on the surface of collagen gels underwent a dramatic reorganization when covered with a second collagen layer. The sequential changes of the subconfluent monolayer in the presence of 10 ng/ml bFGF and 10 μg/ml heparin are illustrated in Fig. 4B. Collagen overlay caused a progressive retraction of the monolayer, which resulted in the appearance of a network of branching and anastomosing cords of the cells. When
Table 2. Effects of 10 ng/ml bFGF and/or 10 μg/ml heparin on tube formation of the cultures of LEC in a three-dimensional collagen gel.

<table>
<thead>
<tr>
<th>Group</th>
<th>Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No agent (control)</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Heparin</td>
<td>56.8±7.3*</td>
</tr>
<tr>
<td>bFGF</td>
<td>72.6±7.1*</td>
</tr>
<tr>
<td>bFGF+heparin</td>
<td>197.7±15.1*</td>
</tr>
</tbody>
</table>

The values are mean ± standard error of the mean (n=16). *p<0.01 vs. the control; **p<0.01 vs. the bFGF group.

Table 3. Effects of 10 ng/ml bFGF and/or 10 μg/ml heparin on the collagen gels induced tube formation of the cultures of LEC isolated from dog thoracic ducts.

<table>
<thead>
<tr>
<th>Group</th>
<th>Length (mm)</th>
<th>Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No agent (control)</td>
<td>63.9±7.2</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>Heparin</td>
<td>120.8±9.6*</td>
<td>5.1±0.5*</td>
</tr>
<tr>
<td>bFGF</td>
<td>202.4±11.8*</td>
<td>9.9±0.6*</td>
</tr>
<tr>
<td>bFGF+heparin</td>
<td>269.7±9.7*</td>
<td>13.2±0.6*</td>
</tr>
</tbody>
</table>

The values are mean ± standard errors of the mean (n=16). *p<0.01 vs. the control; **p<0.01 vs. the bFGF group.

stained with Giemsa, most of these cords showed a central, translucent cleft along their axes, which suggested lumen formation. The effects of 10 ng/ml bFGF and/or 10 μg/ml heparin on the collagen gel-induced tube formation of the LEC (lymphangiogenesis) are summarized in Table 3.

Electron microscopic observation of tube formation of LEC

An examination of ultrathin sections cut perpendicularly to the culture plane sandwiched with collagen gels containing 10 ng/ml bFGF demonstrated clearly that virtually all the endothelial cells delimited a narrow lumen, thus forming a complete tubular structure resembling lymph capillary (Fig. 5). The endothelial tube consisted of three contiguous cells whose plasma membranes had focal points of fusion (Fig. 5). The adjacent cells were extensively overlapped or they formed interdigitations (Fig. 5). The tubes lacked a defined basal lamina.

DISCUSSION

In the present study we used a modification of methods described by Gnepp and Chandler [18] for the isolation, growth, characterization, and long-term culture of LEC. When plated on the gelatin-coated dishes containing MEM complete culture medium, the cells grew as a monolayer of closely apposed cells. As the cultures reached confluence, they exhibited a cobblestone appearance, being characteristic of endothelial cells in vitro. These findings are completely compatible with the observation obtained with bovine or sheep mesenteric lymph vessels [22–24].

The examination of these cells by using immunofluorescence methods to detect Factor VIII related antigens [25] showed that all cells were positive for Factor VIII. The finding strongly supports the conclusion that contamination-free cultures of LEC have been achieved in the present study. This observation also agrees with findings obtained with the other LEC in vitro [22–24]. A pavement pattern of the cells demonstrated by using the silver nitrate staining (Fig. 1) also confirmed the conclusion that the cells cultured in the present study were lymphatic endothelial cells. We also used the Dil-Ac-LDL in the present study as an aid in histological identification of endothelial cells [26]. A marked phagocytosis of Dil-Ac-LDL in the cultured cells may also agree with the conclusion that the cells are lymphatic endothelial cells.

The bFGF-mediated proliferation and migration, and invasion and tube formation of the LEC isolated from dog thoracic ducts. The bFGF at a concentration of 10 ng/ml significantly increased the number of low-density LEC and facilitated migration of the cultured LEC. The bFGF has been known to be a potent mitogen for mesoderm-derived cells [27] and to be capable of triggering the proliferation of vascular endothelial cells at very low concentrations [28]. It also promotes the growth of subconfluent vascular endothelial cells and extends their life spans in long-term cultures [29]. It is known that bFGF stimulates migration, plasminogen activa-
tor synthesis, and DNA synthesis, and bFGF is presumed to be involved in the angiogenesis of blood vessels as an autocrine growth factor [10, 20]. These actions of bFGF may have contributed in the present study to the bFGF-mediated induction of proliferation and migration of the low-density LEC isolated from dog thoracic ducts.

In the present study, bFGF (10 ng/ml) also stimulated invasion and tube formation of the LEC, which had been cultured on the surface of three-dimensional collagen gels. Furthermore, the lymphatic capillary-like tube formation was produced when the LEC were sandwiched between two layers of type I collagen gel. The findings may be compatible with the evidence that type I collagen gels have formed lymphatic capillary-like channels from confluent monolayers of LEC from bovine and sheep mesenteric lymph vessels [30]. The type I collagen gel-mediated tube formation of the LEC isolated from dog thoracic ducts was also accelerated by additional treatment with 10 ng/ml bFGF in the present experiments. The findings suggest that the lymphatic endothelial cells isolated from dog thoracic ducts, similar to endothelial cells isolated from bovine mesenteric lymphatics, have a potential ability to induce lymphatic capillary-like tube formation via facilitated proliferation, migration, and invasion of the LEC in response to bFGF. This may be the first demonstration of bFGF-mediated lymphangiogenesis of the LEC isolated from dog thoracic ducts, in which passive driving forces such as changes in intrathoracic pressure and arterial pulsation play an important role in lymph transport [31].

On the other hand, Pepper et al. [4] clearly demonstrated that lymphatic endothelial cells isolated from bovine mesenteric lymphatics with spontaneous contractions in which active driving forces play a significant role in lymph transport [6] formed capillary-like tube structures in response to bFGF or vascular endothelial growth factor (VEGF). In conclusion, two representative kinds of lymphatic endothelial cells are able to induce in vitro neovascularization of lymph vessels in response to angiogenic cytokines, such as bFGF and VEGF, in a manner similar to what has previously been observed with endothelial cells derived from the blood vascular system [10, 20, 32].

**Facilitated effects of heparin on the bFGF-mediated lymphangiogenesis of the LEC.** Heparin-like molecules, such as heparin and heparan sulfate, found on virtually all cell surfaces determine physiological properties of extracellular matrix [33]. These molecules bind several angiogenic cytokines and modulate their functions either by stabilizing them or by controlling their bioavailability [34]. They also act as low-affinity receptors on cell surfaces and facilitate growth factor activity and receptor binding [13, 35].

In this study, heparin at a concentration of 10 μg/ml caused a significant potentiation of the bFGF-mediated proliferation and migration of the low-density cultures of LEC isolated from dog thoracic ducts. The facilitated effect of heparin on the migration of LEC was observed dose-dependently. Furthermore, heparin at a concentration of 10 μg/ml also potentiated significantly the bFGF-mediated tube formation (lymphangiogenesis) of the LEC when they were incubated between two layers of type I collagen gels. These findings suggest that heparin may have a role in modulating proliferation, migration, and lymphatic capillary-like tube formations of the LEC cultures. The conclusion may be supported strongly by the evidence that heparinase inhibits growth factor mediated proliferation of vascular endothelial cells and thus neovascularization [17].

On the other hand, the glycosaminoglycan heparin inhibits growth and migration of cultured vascular smooth muscle cells [36] and prevents the formation of neointima following vascular injury in rats [37], but it fails to do so in primates [38]. These mechanisms of how heparin mediates its antiproliferative effects have not been defined, although many effects of heparin have been described, including the altered expression of matrix molecules [39], the inhibition of proteases [40], the repression of the early genes c-fos and c-myc [41], and the inhibition of mitogen-activated protein kinase (MAPK) activation by serum [42]. There is a marked controversy over the involvement of heparin in proliferation and migration between endothelial cells and vascular smooth muscle cells.

Thus the present findings of the cultured lymphatic endothelial cells show a marked contrast to the evidence that heparin inhibits proliferation of vascular smooth muscles by preventing the activation of MAPK kinase (MAPKK)-1 [15]. The only MAPK-activating kinase found in the vascular smooth muscle cells was MAPKK-1. Daum et al. [15] also demonstrated that heparin inhibited the activation of the MAPK in baboon vascular smooth muscle cells by serum, but not by platelet-derived growth factor (PDGF). When bFGF was used, heparin had a stimulatory effect on the MAPK. Further investigation will be needed to examine the interaction between heparin and MAPK in the cultured lymphatic endothelial cells in order to give precise knowledge of the molecular mechanisms of heparin.

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bFGF-Mediated Lymphangiogenesis and Heparin


