Angiogenesis and Fibroblast Growth Factors (FGFs) in a Three-Dimensional Collagen Gel Culture

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Summary: Small pieces of mouse aorta were cultured in collagen gels, and the formation of capillary-like tubes from the aortic explant was observed under phase-contrast and transmission electron microscopes. Migration of fibroblastic cells from the aortic explant occurred in 2 days. After about 10 days of culture, capillary-like tubes from the aortic explant were formed in the collagen gels. An immunohistochemical study on the collagen gel culture revealed the expression of fibroblast growth factor 2 (FGF-2) near the aortic explant at the active initial stage, with random migration of fibroblastic cells expressing FGF-9. mRNA was isolated from these cultures, and reverse transcription-polymerase chain reaction (RT-PCR) of the cultures revealed the expressions of both FGF-2 and FGF-9. Based on our results, we propose that FGF-9 is also related to angiogenic events.

Angiogenesis, the development of vessels from preexisting vessels, is essential for a variety of physiologic processes, including organ development (Adams *et al.*, 1999; Erlebacher *et al.*, 1995), wound healing (Nissen *et al.*, 1998), and reproduction (Tamura and Greenwald, 1987). Several techniques have been developed for *in vitro* studies on angiogenesis. In experiments from the early 1980's, researchers found that they could induce the formation of capillary-like tubes by embedding pure endothelial cells in collagen gel (Folkman and Haudenschild, 1980; Montesano *et al.*, 1983). Later, researchers induced angiogenesis in tissues with the use of fibrin and collagen gels (Montesano *et al.*, 1985), as well as matrigel, collagen, fibrin, and plasma clot (Nicosia and Ottinetti, 1990a). Among these various techniques, the culture of blood vessels in collagen gels has been reported to yield relatively steady results (Mori *et al.*, 1988; Nicosia and Ottinetti, 1990b; Akita *et al.*, 1997a, b).

A variety of growth factors are expressed at the site of angiogenesis. The fibroblast growth factors (FGFs) are a family of heparin-binding proteins involved in many biological processes in both developing and adult mammals, including cell prolifera-

tion, differentiation, and angiogenesis (Cancilla *et al.*, 2001). The actions of the FGFs, particularly the basic form (basic FGF = FGF-2), have been described in a large number of cells (for a review see Bikfalvi *et al.*, 1997). In this study, aortic explants from mice were cultured in collagen gels. Examination of these cultures by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry revealed the expressions of both FGF-2 and FGF-9. FGF-2 is well known as an angiogenic factor. We propose that FGF-9 also relates to angiogenic events in the collagen gel culture.

Materials and Methods

Collagen gel culture

This culture technique has already been described by Akita *et al.* (1993, 1997a, b). Thoracic aortae were obtained from 1-month-old ICR male mice. After separating the tunica adventitia from the aorta under a stereoscopic microscope, the blood vessel specimens were cut into small pieces of 3–5 mm in length, placed at the bottom of a tissue culture plate (Falcon 35/3047), overlaid with an

even layer of reconstituted collagen solution (0.3% Cellmatrix type IA, Nitta Gelatin), and allowed to gel at 37°C for about 10 min. After the gels had formed, they were overlaid with Ham's F-12 medium (Bioproducts) containing 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, 100 Units/ml penicillin, and 100 mg/ml streptomycin (Bioproducts), and cultured for 2 weeks in an incubator (95% air/5% CO₂). The culture medium was replaced halfway through the incubation, at 1 week.

Electron microscopy

The cultured materials were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 1 hour, and fixed in 0.1 M phosphate buffer (pH 7.2) containing 1% OsO₄ for another hour. The specimens were then dehydrated with ethanol, embedded in epoxy resin, cut into ultrathin sections, and stained with uranyl acetate and lead citrate. The stained sections were observed under a transmission electron microscope (Hitachi H-7000).

RT-PCR

The following methods were used to isolate mRNA and prepare cDNA for the RT-PCR analysis. The expressions of nine FGF family genes (FGF-1, -2, -3, -4, -5, -6, -7, -8, -9) were analyzed. We adopted several conditions from the method developed by Ozawa *et al.* (1997), including the PCR primers, pH and Mg²⁺ concentration, annealing temperature, and number of cycles for amplification of FGF and GAPDH (as a control) cDNAs.

1. Grind cells migrating from the aortic explant into a mortar on liquid nitrogen. Add Lysis/Binding Buffer (μ MACS mRNA Isolation Kit; Daiichi Chemical).
2. Apply the sample on top of the Lysate Clear Column.
3. Add Oligo (dT) Micro Beads to the tissue.
4. Place a MACS Column Type μ in the magnetic field.
5. Apply tissue from Lysis/Binding Buffer.
6. Apply tissue lysate on top of the column matrix, and let the lysate pass through (magnetically labeled mRNA is retained in the column).
7. Rinse the column with Lysis/Binding Buffer to remove proteins and DNA.
8. Rinse the column with Wash Buffer to remove rRNA and DNA.
9. Apply pre-heated Elution Buffer for elution of the mRNA.
10. Perform the first-strand cDNA synthesis from the template RNA (*Ready-To-Go; You-Prime First-Strand Beads; Pharmacia*).
11. RT-PCR.

Thermal cycling reaction using a DNA thermal

cycler device (Takara PCR Thermal Cycler MP): The thermocycling program consisted of a 94°C hot start, followed by 35 cycles of 94°C for 1 min, 55–70°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. RT-PCR products electrophoresed through 1% agarose gels were visualized by ethidium bromide staining and UV transillumination.

Immunohistochemistry

Factor VIII-related antigen (von-Willebrand-factor): The gels were detached from the culture dish with a spatula, placed evenly on a glass slide, covered with a lens tissue and filter paper, and pressed for 1 min (Mori *et al.*, 1988). After gently removing the papers, the gels were air-dried and used for immunostaining for factor VIII-related antigen. The dried gels were fixed with acetone for 5 min. After pretreatment with normal serum, they were washed with phosphate-buffered saline (PBS), incubated with goat anti-human factor VIII-related antigen (Biomedica Corp., USA) for 60 min, washed with PBS, reincubated with biotinylated goat IgG for 20 min, washed and incubated with peroxidase avidin, and stained with AEC and 0.02% H₂O₂ in PBS. Gels incubated with normal goat serum instead of anti-human factor VIII-related antigen antibody were used as control.

FGF-2 and FGF-9: The gels were detached from the culture dish with a spatula, fixed in 10% formalin for 1 hour, pretreated with normal serum, and washed with PBS. For immunocytochemistry, the gels were incubated for 5 min with 3% hydrogen peroxide to suppress endogenous peroxidase activity. Next, they were incubated overnight at 4°C with the one of the following antibodies: anti-human FGF-2; 147, sc-79 (this antibody reacts with FGF-2 of mouse, rat and human origin; Santa Cruz Biotechnology) or anti-mouse FGF-9 (Antigenix America). After washing with PBS, the primary antibody was detected with streptavidin-biotin by the DAKO LSAB2 kit according to the manufacturer's instructions. The specimens were then treated by sequential 15-minute incubations with the biotinylated link antibody and peroxidase-labeled streptavidin. The last stage of staining was a 5-minute incubation with the substrate-chromogen solution (AEC and 0.02% H₂O₂ in PBS). Gels incubated with normal rabbit serum instead of antibodies were used as controls.

Results

Light and electron microscopy

Spindle-shaped cells began migrating from the aortic explant after 2 days of culture. Three to four

Fig. 1. a. Phase-contrast micrograph of the migrating cells into the collagen gels. After 5 days in culture, cell migration occurred from the end of the aortic explant (asterisk). Capillary-like tubes are not formed at this time. $\times 100$
b. Electronmicrograph of the migrating cells into the upper layer of collagen gels. These cells exhibited fibroblastic characteristics in the morphology. $\times 5,500$

layers of what morphologically resembled fibroblastic cells were overlaid on the superficial and bottom layers of the collagen gels, and migrating cells were also seen in the middle layer. However, no tubular structure was visible at this time (Fig. 1a, b). The formation of capillary-like tubes first appeared after about 10 days of culture (Fig. 2a). Formations of capillary-like tubes consisting of 2–3 cells each were found in the collagen gels. Weibel-Palade bodies were observed in the cells (Fig. 2b).

RT-PCR

FGF-2 expression was observed at the active initial stage, with random migration of fibroblastic cells from the aortic explant, before the capillary-like tube formation. RT-PCR did not detect any FGF-9 expression at this time. Once the capillary-like tube formation was underway, expressions of both FGF-2 and FGF-9 were observed (Fig. 3, 4).

Immunohistochemistry

Capillary-like tubes were positive for the immunostaining of factor VIII-related antigen (Fig. 5). FGF-2 expression was observed in the fibroblastic cells migrating from the aortic explant at the active initial stage (Fig. 6a), but the expression grew indistinct with time. The capillary-like tubes were negative for the immunostainings of FGF-2 and FGF-9. The migrating fibroblastic cells also expressed FGF-9. After capillary-like tube formation, the FGF-9-positive cells were dominant (Fig. 6b).

Discussion

To study the process of angiogenesis and test new agents with (anti)-angiogenic potential, suitable assays are essential (Deckers *et al.*, 2001). The most commonly used *in vivo* assays are the corneal micropocket assay, the chorioallantoic membrane (CAM) assay, and the dorsal skin-fold assay (Fournier *et al.*, 1981; Jakob *et al.*, 1978; Kenyon *et al.*, 1996). The corneal micropocket assay, an analysis conducted within an avascular environment, is often used to study the efficacy of angiogenic compounds. The efficacy of anti-angiogenic compounds in inhibiting growth factor induced-vascularization and spontaneous vascularization is usually studied in vascular environments such as the CAM assay or the dorsal skin-fold assay. These assays have proven useful and have dramatically advanced our knowledge of angiogenesis. However, they are also limited in several respects (for a detailed review see Jain *et al.*, 1997): (a) complicated surgical techniques are required; (b) only a limited number of test compounds can be assayed (in the case of the micropocket assay, for example); and (c) simultaneous assessment of both angiogenic and anti-angiogenic compounds in the same assay is not feasible without the addition of exogenous growth factors. Fortunately, the method described here is free from these limitations. Fujita *et al.* (2002) used this culture method to test angiogenic and anti-angiogenic agents (TNF- α , thalidomide). The *in vivo* mechanism of angiogenesis is very complex, involving not only endothelial cells but also other cellular components such as pericytes,

Fig. 2. a. After 14 days in culture, many capillary-like tubes are seen surrounding the aortic explant (asterisk). Giemsa stain, $\times 100$
 b. An electronmicrograph of the capillary-like tubes after 14 days in culture. Arrows indicate the site of junctional complex. The cells are connected to one another by the junctional complex. Pinocytotic vesicles have not yet formed. Multiple cisternae of the rough-surfaced endoplasmic reticulum, mitochondria, and free ribosomes are distributed throughout the cytoplasm. CL, capillary lumen. $\times 5,500$

Fig. 3. RT-PCR of FGF cDNAs. Nine FGFs were analyzed. After 14 days in culture, the expressions of FGF-2 (lane 2) and FGF-9 (lane 9) were detectable in the capillary-like tubes and in the cells that had migrated from the aortic explant. Lane M is a molecular marker.

smooth muscle cells, fibroblasts, and macrophages. Extracellular matrix, angiogenic factors, and adhesive molecules (integrins) also play important roles in angiogenesis. Compared with the techniques using only endothelial cells, the *in vitro* model for the

study of angiogenesis described here permits the observation of angiogenesis within more of an *in vivo* environment. In the present study, we initially observed fibroblastic cells actively migrating from the aortic explant, and this was followed by

Fig. 4. RT-PCR of FGF-2 and FGF-9 cDNAs.

- a. FGF-2, after 5 days in culture. FGF-2 expression was detectable, but capillary-like tube formation was not observed.
- b. FGF-2, after 14 days in culture. FGF-2 expression was detectable, and capillary-like tubes were formed.
- c. FGF-9, after 5 days in culture. FGF-9 expression was not detectable, and capillary-like tubes were not formed.
- d. FGF-9, after 14 days in culture. FGF-9 expression was observed. Lane M is a molecular marker.

Fig. 5. Immunostaining using anti factor VIII-related antigen antibody. After 14 days in culture, the reddish brown color of the newly formed capillary-like strand indicates positive staining for antibody to factor VIII-related antigen (arrowheads). Nuclei were stained dark blue with hematoxylin. $\times 175$

Fig. 6. a. Immunostaining using anti FGF-2 antibody. After 5 days in culture, migrating cells from the aortic explant showed positive reaction. Capillary-like tubes were not formed. The aortic explant located in the upper left corner. $\times 175$
b. Immunostaining using anti FGF-9 antibody. After 14 days in culture, migrating cells from the aortic explant showed a positive reaction. Arrowheads indicate widely spread cells grown on the bottom of the culture dish. Arrows indicate cells with fine processes. $\times 175$

the formation of capillary-like tubes. Brown *et al.* (1996) also observed the formation of capillary-like tubes from a placental blood vessel stump embedded in fibrin gel, but they turned their focus away from this finding in order to investigate the migration of fibroblasts preceding the formation of capillary-like tubes. Deckers *et al.* (2001) described the use of fetal mouse metatarsals to study the formation of capillary-like tubes. In their experiments, the formation of PECAM-1-positive tube-like structures was observable from day 7, after a feeder layer of fibroblast-like cells had been formed. To explain the time lag, they speculated that the endothelial cells might have required the feeder layer of fibroblastic cells as a pathway along which to migrate. Our present findings suggest that migration of fibroblastic cells before the formation of capillary-like tubes is quite an important event for angiogenesis. These fibroblastic cells act as a feeder layer and may produce various angiogenic factors.

Angiogenesis will not take place without angiogenic growth factors, including FGF-2 (Montesano *et al.*, 1986), FGF-2 and VEGF (Folkman and Shing, 1992). Angiogenic growth-regulatory molecules are thought to be inducers of mitogenesis in vascular endothelial cells, as the formation of new vessels depends on endothelial proliferation. VEGF is a growth factor that acts specifically on vascular endothelial cells. Gerber *et al.* (1999) suggested that VEGF is a key regulator of angiogenesis during bone development. In a study using fibrin gel, Brown *et al.* (1996) found that both VEGF and FGF-2 mRNAs were expressed in the cells migrating from the placental blood vessel stump. In our previous experiment, VEGF promoted cell outgrowth from aortic explant (Akita *et al.*, 1996). According to Cancilla *et al.* (2001), fibroblast growth factors (FGFs) are a family of at least 21 heparin-binding proteins involved in many biological processes including angiogenesis. FGF-2, a potent angiogenic molecule *in vivo* and *in vitro*, stimulates endothelial cell growth (Moscatelli *et al.*, 1986; Presta *et al.*, 1986; Leach *et al.*, 1997), smooth muscle cell growth, wound healing, and tissue repair (Basilico and Moscatelli, 1992; Schwartz and Liaw, 1993). This FGF prototype has pleiotropic effects in different cell and organ systems. Kuzuya *et al.* (1999) and Satake *et al.* (1998) reported that FGF-2 plays important roles in angiogenesis. When fetal bovine aortae (BAECs) were embedded in collagen gels, they underwent apoptotic death within 2 days unless the cultures were repeatedly supplied with FGF-2. Supplementation with FGF-2 induced endothelial cell differentiation, resulting in capillary-like tube formation inside collagen gels. Endothe-

lial cells derived from BAECs undergo apoptosis in three-dimensional (3-D) type I collagen gels in the absence of FGF-2. In the presence of FGF-2, BAECs survive and form a capillary-like tube structure in 3-D culture. In the study by Brown *et al.* (1996) using fibrin gel, FGF-1 mRNA was also expressed in the cells migrating from the placental blood vessel stump. In the present study, we could not detect the expression of FGF-1 mRNA. FGF-1, like its relative FGF-2, stimulates angiogenesis both *in vitro* and *in vivo* (Maciag, 1990). However, FGF-1 is not significantly expressed in endothelial cells, suggesting a paracrine mechanism of action. Smooth muscle cells express FGF-1, and this expression may be involved in the induction of neovascularization in atherosclerotic lesions (Maciag, 1990; Nabel *et al.*, 1993). In the RT-PCR and immunohistochemical investigations of our present study, the expression of FGF-9 was dominant within the FGF family. FGF-9, glia-activating factor originally cloned from human glioma cells, stimulates the cell growth of fibroblasts and glial cells and is expressed in the brain and kidney of adult rat (Naruo *et al.*, 1993; Miyamoto *et al.*, 1993). The biological roles of FGF-9 are not well known. Pilcher *et al.* (1997) and Miyagi *et al.* (1998) reported that FGF-9 relates to the expression of matrix metalloproteinases (MMPs) and their inhibitors through the FGF cell-surface receptor (FGFR). Haas *et al.* (1998) hypothesized that MMPs play a key role in the processes of endothelial cell migration and matrix remodeling during angiogenesis. They reported that three-dimensional type I collagen gels induced coordinate expression of matrix metalloproteinases MMP-2 and MT1-MMP (membrane type I-MMP) in microvascular endothelial cells. Further studies of MMP and FGFR are needed to clarify the angiogenic roles of FGF-9. FGFs mediate essential cellular functions by activating one of four alternatively spliced FGFRs (Chellaiah *et al.*, 1999). Since FGF-9 and other members of the FGF family can bind to the same receptors as FGF-2, they may also prove to be angiogenic, if expressed at appropriate levels in specific sites. In this study, we propose that FGF-9 works alongside FGF-2 in inducing angiogenesis *in vitro*.

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