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ORIGINAL ARTICLE

FGF-2 but not FGF-1 binds fibrin and supports prolonged endothelial cell growth

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Summary. Endothelial cell viability and growth are dependent on both polypeptide growth factors, and integrin-mediated matrix interactions. We have now examined the ability of fibrin-binding and non-binding growth factors to support long-term endothelial cell growth in the presence or absence of the soluble form. Endothelial cells were cultured on a fibrin surface, with or without FGF-1 or FGF-2, and proliferation was determined by ^{3}H-thymidine incorporation. Cells cultured on fibrin with no growth factor showed minimal proliferation up to 96 h. In contrast, when FGF-2 was incorporated into fibrin, proliferation was increased 6.5 ± 0.6-fold, equal to growth on a fibrin surface with FGF-2 continually present in the medium. Thymidine incorporation was similar when cells were cultured on a fibrin surface that had been incubated with FGF-2 and then the growth factor removed (8.6 ± 0.5-fold). In contrast to results with FGF-2, a surface of fibrin exposed to FGF-1 supported minimal growth, whereas growth was comparable to either FGF-1 or FGF-2 present in the medium. Comparable results were observed when proliferation was quantitated by cell counting at times up to 48 h. Binding studies demonstrated no high-affinity interaction of FGF-1 with fibrinogen or fibrin. We conclude that FGF-2 bound to fibrin supports prolonged endothelial cell growth as well as soluble FGF-2, whereas FGF-1 does not bind to fibrin and can support endothelial cell growth only if continually present in soluble form. Fibrin may serve as a matrix reservoir for FGF-2 to support cell growth at sites of injury or thrombosis.

Keywords: endothelial cells, FGF-1, FGF-2, fibrin.

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Introduction

Endothelial cell responses play an important role in wound healing, inflammation and tumor growth, and their properties are also influenced by interactions with fibrinogen and fibrin which can support adhesion [1], stimulate protein synthesis and secretion [2,3], alter inflammatory cell interactions and facilitate angiogenesis [4]. Endothelial cells are physiologically stable when exposed to a high fibrinogen concentration in blood, but marked phenotypic changes occur with fibrin interaction, including loss of monolayer organization [5], migration [6,7], interleukin-8 expression [8], synthesis and secretion of tissue-type plasminogen activator [9], plasminogen activator inhibitor type-1 [9,10], and von Willebrand factor (VWF) [3]. These responses require the thrombin-induced cleavage of fibrinopeptide B from the fibrinogen Bβ chain, which results in exposure of a reactive site at new amino terminus of fibrin β chain that interacts with VE-cadherin on endothelial cells [11,12].

The cell responses that accompany fibrin formation are initiated and regulated by a variety of polypeptide growth factors including the family of FGFs. Previous studies indicate that FGF-1 and FGF-2 play important roles in modulating vascular responses by increasing endothelial cell proliferation [13,14] and stimulating migration [15,16], as well as increasing secretion of collagenase [17], urokinase plasminogen activator [18], urokinase plasminogen activator receptor [19] and plasminogen activator inhibitor type-1 [20], proteins that are important in the degradation of extracellular matrix. FGFs also influence endothelial cell adhesion through altering the expression of integrin receptors [21]. Although FGF-1 and FGF-2 lack a signal sequence for secretion, they are released from vascular cells following injury, are active in the pericellular environment [21], and FGF-2 mRNA is upregulated in atherosclerotic arteries [22] and following vessel injury [23]. The activities of FGFs are mediated by their binding to high-affinity tyrosine kinase receptors present at the cell surface [24] and further by interactions with heparan sulfate proteoglycans [25,26]. Limited evidence suggests that cell proliferation *in vitro* is dependent on the continuous presence of growth factor in the culture medium. For example, Zhan *et al*. [27] showed that initiation of DNA synthesis required exposure of fibroblasts to growth factor in the medium for a minimum of 12 h. Also, Presta *et al*. [28] demonstrated that

bovine aortic endothelial cells required 12–14 h of exposure to FGF-2 in culture medium before commitment to proliferate [27–31]. Longer-term studies of comparing growth with soluble and matrix-bound growth factor are not available.

We have shown previously that FGF-2 binds to fibrin(ogen) with high affinity [32]. The need for fibrin to support endothelial cell spreading, migration and proliferation and the stimulation of similar responses by FGF-1 and FGF-2 suggest that these processes may be interrelated. This concept is supported by evidence that fibrin clots are a good matrix to support FGF-stimulated angiogenesis *in vitro* [33,34]. We therefore postulated that fibrin(ogen) could act as a reservoir of growth factor to support long-term endothelial growth and tested this hypothesis by comparing endothelial cell growth with soluble or fibrin-bound FGF-1 and FGF-2.

Materials and methods

Fibrinogen and fibrin preparation

Human fibrinogen was obtained from Enzyme Research Laboratories (South Bend, IN, USA) and copurifying fibronectin was removed by gelatin–Sepharose chromatography [35]. Residual fibronectin remaining after gelatin–Sepharose chromatography was further depleted by immunoaffinity chromatography as described elsewhere [1]. The fibronectin concentration was determined by ELISA (American Diagnostica, Greenwich, CT, USA) and represented $<0.02\%$ of the total protein. Fibrin-coated wells were prepared using 1 mg mL^{-1} of fibrinogen in McCoy's 5A medium to which 1 U mL^{-1} of thrombin (Calbiochem-Novabiochem, La Jolla, CA, USA) was added, mixed and rapidly pipetted into 24-well non-tissue culture-treated cell culture plates. The solution was aspirated after 45 s and before polymerization, leaving a thin coating of fibrin on the surface. Wells coated with fibrin with FGF-1 or FGF-2 were prepared in the same way except that 100 ng mL^{-1} of FGF-1 or FGF-2 (Peprotech Inc., Rocky Hill, NJ, USA), was added to the fibrinogen and thrombin solution prior to coating wells. In some conditions, FGF-1 or FGF-2 was added after the surface had been coated with fibrin. Fibrin-coated wells were treated with 1 μg mL^{-1} of D-phenylalanyl-L-prolyl-L-arginyl-chloromethyl ketone (Bachem, Torrance, CA, USA), a synthetic specific thrombin inhibitor, for 30 min to inhibit any remaining thrombin, and this was followed by two washes with McCoy's 5A medium before plating the cells.

Cell culture

Primary endothelial cells were obtained from human umbilical veins. Cells were seeded on 0.2% w/v gelatin-coated 25-cm^2 tissue culture flasks and cultured in McCoy's 5A medium (Flow Laboratories, McLean, VA, USA) containing 20% fetal bovine serum (FBS), 50 μg mL^{-1} endothelial cell growth supplement (ECGS) (Collaborative Research, Inc., Bedford, MA, USA) and 100 μg mL^{-1} heparin (Sigma Chemical Co., St Louis, MO, USA) until they reached confluence, typically within 4–5 days. Cells were passaged up to two times before use and then placed in suspension by trypsinization of monolayers. Cells were suspended by rinsing in Hank's balanced salt solution followed by brief incubation with trypsin–EDTA (Gibco Life Technologies, Inc., Grand Island, NY, USA). The cells were pelleted by centrifugation for 10 min at 500 × *g* and resuspended in McCoy's 5A medium in the absence of serum. This wash procedure was repeated twice prior to use in experimental protocols.

^{3}H-thymidine incorporation

Approximately 2×10^4 endothelial cells suspended in McCoy's 5A medium were plated on fibrin-coated 12-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) with or without FGF in the medium. For some experiments FGF was combined with fibrinogen prior to addition of thrombin and in others FGF was added to wells after fibrin formation and cells were allowed to grow for 4 days without the addition of growth factor. ^{3}H-thymidine (1 μCi mL^{-1}) (New England Nuclear, Boston, MA, USA) was added to the culture medium 4 h prior to harvest to determine ^{3}H-thymidine incorporation. Every 24 h, nonadherent cells were removed by washing twice with ice-cold phosphate buffered saline (PBS). To each well was then added 500 μL of 10% ice-cold trichloroacetic acid (TCA), and precipitates were collected on a filter using a filtration manifold. Filters were washed twice with ice-cold 5% TCA, followed by 95% ethanol, allowed to air dry and then suspended in scintillation fluid. Acid precipitable counts per minute (cpm) were quantitated using a scintillation counter.

Cell proliferation

Fibrin-coated wells were prepared as described above. FGF-1 or FGF-2 (100 ng mL^{-1}) was added to the wells and incubated for 30 min at 37 °C for 30 min. Wells were then washed with McCoy's 5A medium three times to remove unbound growth factor. Approximately 1×10^4 endothelial cells suspended in McCoy's 5A medium were plated in the wells and incubated for various times. The cells were then trypsinized and counted using a hemocytometer.

Propidium iodide staining of endothelial cells

Cells were cultured on 1 mg mL^{-1} fibrin surface prepared on Thermanox® coverslips (Marsh Biomedical Co., Rochester, NY, USA) with FGF-1 or FGF-2 for 24 h. Cells were then fixed with 3.7% formaldehyde in PBS for 20 min, washed three times with PBS, permeabilized with 0.5% Triton X-100 for 20 min and then washed again three times with PBS. They were then mounted on glass microscope slides using gel mount (Gel/Mount®; Birmedia Corp., Foster City, CA, USA). Propidium iodide counterstain (3 μg mL^{-1} in PBS) was applied and covered with a glass coverslip, the edges were sealed using rubber cement, and the slides were stored at −20 °C. Cells were viewed using a Nikon Eclipse E-800 fluorescence microscope equipped with a dual wavelength filter cube.

Statistical analysis

Each experiment was performed at least three times, and either triplicate or quadruplicate wells were used in each experiment. The significance of differences in means was determined using a two-tailed Student's *t*-test. Variance is described as ±SD.

Results

Human umbilical vein endothelial cells were cultured on a fibrin surface with or without FGF-2, cells were pulsed with ^{3}H-thymidine 4 h prior to harvesting, and proliferation was measured by isotope incorporation at times up to 96 h. Overall, proliferation declined from 24 to 72 h and then remained constant until 96 h (Fig. 1). Cells cultured with no added growth factor showed minimal proliferation at any time, and few cells were present at 96 h. In contrast, when FGF-2 was incorporated into fibrin, cell proliferation remained high from 24 to 96 h. At 96 h, ^{3}H-thymidine incorporation was 6.5 ± 0.6-fold greater than medium alone ($P < 0.01$), and this was the same as growth on a fibrin surface with FGF-2 present continually in the medium. There was no significant additional growth when FGF-2 was both incorporated into the fibrin and also present in the medium during incubation (7.2 ± 0.6-fold). Growth was similar when cells were cultured on a fibrin surface that had been incubated for 30 min with FGF-2 and then the growth factor removed (8.6 ± 0.4-fold) at 96 h.

To compare results with FGF-2 and FGF-1, we first investigated potential binding of FGF-1 to fibrinogen using soluble ^{125}I-radiolabeled FGF-1 and fibrinogen immobilized on Sepharose beads. There was saturable and specific binding with FGF-2 as previously demonstrated [32], but no binding was observed with FGF-1 (data not shown). We then compared the cell proliferation in the presence of FGF-1 or FGF-2 with or without added fibrinogen. Morphologically cells were sparse without growth factor or in the presence of fibrinogen alone (Fig. 2A). Addition of FGF-1 or FGF-2 increased growth, and fibrinogen potentiated the proliferation with FGF-2 but not FGF-1. Cell growth was quantitated by ^{3}H-thymidine incorporation (Fig. 2B). Proliferation increased 6.2 ± 0.5-fold with 25 ng mL^{-1} FGF-1 and 6.5 ± 0.7-fold with the same concentration of FGF-2 when growth factor was present in the medium without fibrinogen. When 10 μg mL^{-1} fibrinogen was added to the medium, proliferation remained at 6.7 ± 0.4-fold with FGF-1 but increased to 14.9 ± 1.2-fold with FGF-2.

Endothelial cells were also cultured on fibrin in the presence of FGF-1 or FGF-2, and in some conditions growth factors were removed from the medium after 30 min and the cells viewed microscopically (Fig. 3). Cells proliferated well on fibrin with FGF-1 or FGF-2 in the medium (Fig. 3A,B). However, cells grew less when FGF-1 was washed out (Fig. 3C); but growth was not affected when FGF-2 was removed from the medium (Fig. 3D). As quantitated by ^{3}H-thymidine incorporation, cell growth was optimum on fibrin with FGF-1 or FGF-2 in the medium, showing ^{3}H-thymidine incorporation of 4.2 ± 1.3 and 4.8 ± 1.8-fold, respectively, over medium alone (Fig. 4). However, when FGF-1 was washed out after 30 min, cell growth declined significantly ($P < 0.03$). In contrast, when FGF-2 was removed from the medium, cell growth was not affected, as proliferation was 4.6 ± 1.2-fold (Fig. 4).

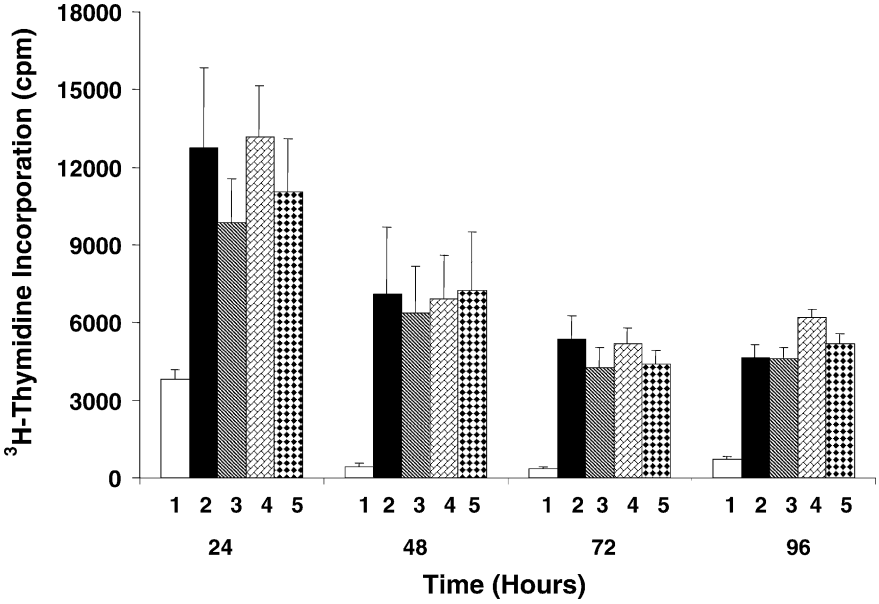

Fig. 1. Effect of fibrin-bound FGF-2 on growth of endothelial cells: endothelial cells were plated on fibrin-coated wells prepared in the presence or absence of FGF-2 in McCoy's 5A medium supplemented with 1% Nutridoma®, with or without 25 ng mL^{-1} of FGF-2 in the medium for various times. ^{3}H-thymidine (1 μCi) was added 4 h before harvesting cells. Isotope incorporated into DNA was precipitated with trichloroacetic acid (TCA), collected by vacuum filtration and measured by scintillation counting. (1) Cells plated on a fibrin surface with no FGF-2 present in the matrix or in the medium. (2) Cells plated on fibrin matrix with FGF-2 present only in the medium. (3) Cells plated on fibrin with FGF-2 and no growth factor present in the medium. (4) FGF-2 was added to preformed fibrin for 30 min, then washed away. (5) Cells plated on fibrin plus FGF-2 and FGF-2 was also present in the medium. The results expressed are the mean ± SD of five separate experiments.

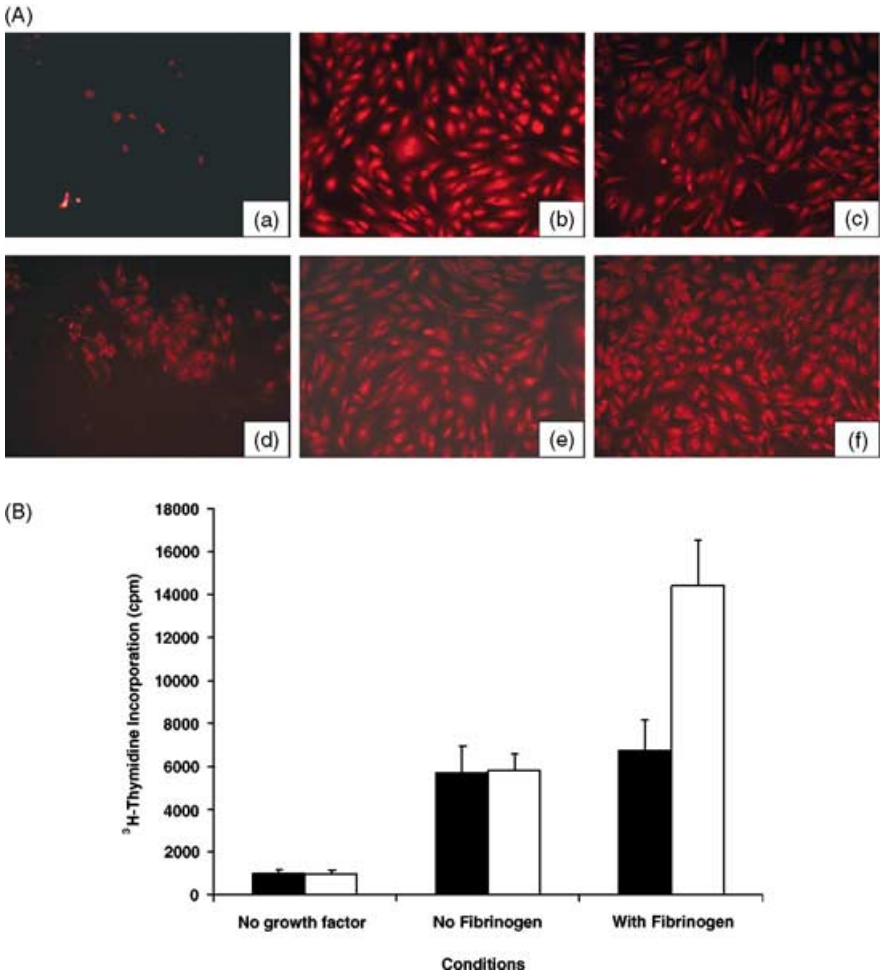

Fig. 2. Endothelial cell proliferation in the presence of FGF-1 and 2. (A) Endothelial cells were plated on gelatin-coated coverslips in McCoy's 5A medium supplemented with 20% fetal bovine serum (FBS), 50 μg mL^{-1} of endothelial cell growth supplement (ECGS) and 100 μg mL^{-1} of heparin and allowed to adhere for 6 h. The cells were then washed twice with McCoy's medium and incubated in serum-free medium containing 1% Nutridoma® with no growth factor (a,d), 25 ng mL^{-1} of FGF-1 (b) or FGF-2 (e), FGF-1 with 10 μg mL^{-1} of fibrinogen (c) or FGF-2 with fibrinogen (f). After 24 h, cells were fixed, permeabilized in 0.5% Triton X-100, washed with phosphate-buffered saline three times, then stained with propidium iodide. The coverslips were viewed under a fluorescence microscope. Bar = 100 μm. (B) Endothelial cells were plated on gelatin-coated wells in McCoy's 5A medium supplemented with 20% FBS, 50 μg mL^{-1} of ECGS and 100 μg mL^{-1} of heparin and allowed to adhere for 6 h. The cells were then washed twice with McCoy's medium and incubated in serum-free medium containing 1% Nutridoma®, 1 μCi of ^{3}H-thymidine with no growth factor, 25 ng mL^{-1} of FGF-1 or FGF-2, with or without 10 μg mL^{-1} of fibrinogen, for 24 h. Black bar and white bar represent FGF-1 and FGF-2, respectively. The results expressed are the mean ± SD of three separate experiments.

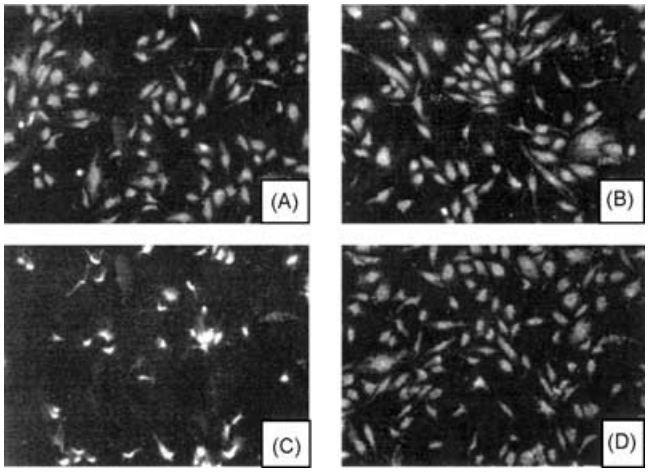

Fig. 3. Cell growth on fibrin surfaces with FGF-2 or FGF-1. Culture wells containing coverslips were coated with 1 mg mL^{-1} of fibrin. Surfaces made with fibrin plus FGF-1 (A); fibrin plus FGF-2 (B); fibrin with FGF-1 washed out after 30 min (C); and fibrin with FGF-2 washed out after 30 min (D). The cells were cultured in McCoy's medium containing no growth factor. After 24 h, cells were fixed, permeabilized in 0.5% Triton X-100, washed with phosphate-buffered saline three times, then stained with propidium iodide. The coverslips were viewed under a fluorescence microscope.

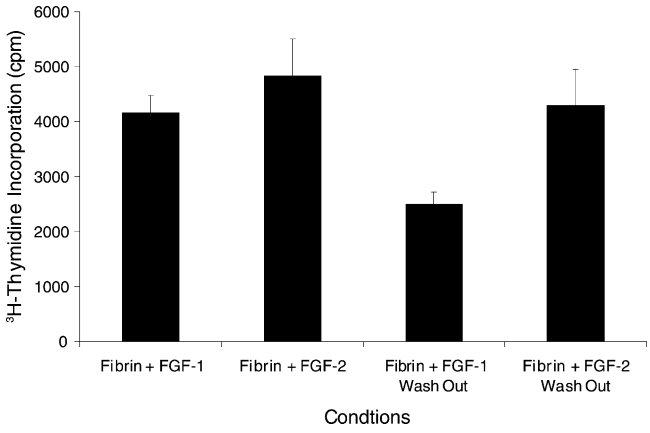

Fig. 4. Endothelial cells cultured on fibrin in the presence of FGF-1 or FGF-2. Endothelial cells were plated on fibrin-coated wells that were then incubated with or without 25 ng mL^{-1} FGF-1 or FGF-2 in McCoy's 5A medium supplemented with 1% Nutridoma® and 1 μCi of ^{3}H-thymidine for 24 h. After incubation with growth factor, some wells were washed repeatedly and then medium containing no growth factor was added ('wash out'). The results expressed are the mean ± SD of three separate experiments.

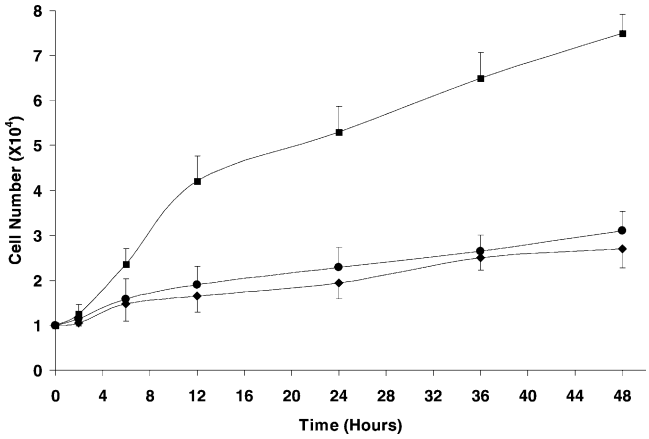

Fig. 5. Growth curve of endothelial cells cultured on fibrin: cell culture wells were coated with fibrin and then incubated in medium (◆), medium with FGF-1 (●) or medium with FGF-2 (■). After 30 min, the medium was removed and the surface was washed to remove unbound FGF. Approximately 1×10^4 endothelial cells suspended in McCoy's 5A medium were then plated on the surface, and the plates were incubated at 37 °C. At selected times, cells were detached by trypsinization and enumerated in a hemocytometer.

Similar results were obtained when proliferation was determined by cell counting. Endothelial cells were cultured on fibrin in the presence or absence of FGF-1 or FGF-2, growth factors were removed from the medium after 30 min and the cell number was counted using a hemocytometer (Fig. 5). Cell growth was minimal on a surface of fibrin alone, or if FGF-1 was washed away after 30 min exposure. In contrast, cell growth continued up to 48 h on a fibrin surface exposed to FGF-2, indicating that bound FGF-2 could support cell growth for a prolonged period of time.

Because fibrin-bound FGF-2 is in equilibrium with free growth factor in solution, additional experiments were conducted to determine if long-term cell growth was due to FGF-2 released from fibrin into the medium. Fibrin was prepared by clotting fibrinogen with thrombin, and 100 ng mL^{-1} radiolabelled FGF-2 was either added to the fibrinogen solution before clotting or incubated with fibrin after polymerization. With both methods, the total FGF-2 bound was 33 ± 4 ng well^{-1} or 8 ng cm^{-2} of fibrin surface. The fibrin was overlaid with medium, and release of radiolabelled growth factor was measured. The concentration of soluble FGF-2 remained stable at 0.4 ± 0.2 ng mL^{-1} in the absence of cells and 0.8 ± 0.1 ng mL^{-1} in the presence of cultured endothelial cells. In the presence of cultured endothelial cells, the concentration increased further and was 0.8 ± 0.1 ng mL^{-1} at 24 h. These concentrations of free FGF-2 are insufficient to support the high level or proliferation observed [36].

Discussion

The results presented demonstrate that fibrin-bound FGF-2 supports long-term endothelial cell growth in the absence of soluble FGF-2. Cell proliferation was sustained for up to 96 h in the absence of FGF-2 in culture medium, and there was no significant difference in growth if FGF-2 was in the medium, fibrin-bound or both, suggesting that the bound, rather than soluble growth factor was dominant in stimulating proliferation. Equilibrium binding ensures that small amounts of bound FGF-2 would dissociate into the medium. This was confirmed experimentally, with results demonstrating a soluble concentration <0.01 nM for prolonged periods. This amount would be insufficient to support cell growth, and is consistent with previously demonstrated high affinity of FGF-2 for fibrinogen and fibrin [32]. FGF-1 and FGF-2, the two prototypes of the FGF family, have 56% sequence homology and similar 3-dimensional structures [37]. Despite this similarity, however, we demonstrated that FGF-1, unlike FGF-2, does not bind fibrinogen. Consistent with lack of binding, the continual presence of FGF-1 in the medium was required to support endothelial cell proliferation in contrast with FGF-2.

This is the first report comparing long-term endothelial cell growth with soluble compared with matrix-bound growth factor. Presta *et al*. [28] found that endothelial cells need at least 12 h of exposure to soluble FGF-2 to enter S phase, but did not examine long-term growth or the effect of matrix binding [28]. Zhan *et al*. [27] found that initiation of DNA synthesis in 3T3 cells required the continuous exposure to soluble FGF-1 for a minimum of 12 h. Interaction of the growth factor with its receptors appeared to be a continuous process, requiring function of a steady-state population of occupied FGF receptors at the cell surface and resulting in continual distribution of exogenous growth factor to both cytosol and nucleus. Receptor phosphorylation, an immediate response to growth factor binding, declined rapidly after removal of FGF-1 from the medium. Results with FGF-2 or with endothelial cells were not reported. Epidermal growth factor and platelet-derived growth factor also require 8–12 h of continuous exposure in soluble form to stimulate maximum DNA synthesis in fibroblast cells [29]. In contrast, the results presented indicate that fibrin-bound FGF-2 retained activity and supported endothelial cell growth for up to 96 h with no additional stimulation provided by soluble growth factor.

The importance of extracellular matrix-bound FGF is particularly relevant to our demonstration that FGF-2 binds specifically to fibrinogen and fibrin with comparable affinities [32]. The implications of this interaction imply a new level of coordination between the hemostatic system and cell regulatory growth factors in the vascular response to injury and angiogenesis. Endothelial cells are exposed to fibrin physiologically and also pathologically in response to vessel injury, where fibrin forms the matrix necessary for cell organization and healing. As such, fibrin may play a role similar to that of extracellular matrix in binding FGF-2 and other growth factors, localizing their action, providing protection from proteolytic degradation [38] and performing a reservoir function. In addition, interactions in regulating cell response may occur through coordination of integrin and growth factor receptor activation. The sensitivity of endothelial cell receptors to growth factor-induced signaling mechanisms is altered by their interactions with matrix glycoproteins through integrin receptors [39]. Studies *in vitro* have demonstrated colocalization of integrin

receptors with high-affinity FGF receptors within the focal adhesion complex [40]. The binding of FGF-2 to the multivalent fibrin(ogen) molecule also has the potential to facilitate the aggregation of growth factor and integrin receptors to promote signal integration.

The activity and distribution of FGFs are also affected by binding to cell-surface heparan sulfate proteoglycans that are thought to be important in localizing and modulating their activities [39]. Although of lower affinity than binding to tyrosine kinases receptors, the association with heparan sulfate is important physiologically in protecting growth factors from proteolytic degradation [41–43] and also providing a local reservoir of growth factor that can be released by enzymes that degrade proteoglycans [44]. Similar to heparin, binding of FGF-2 to fibrinogen potentiates its capacity to stimulate endothelial cell proliferation [36], and fibrinogen protects FGF-2 from proteolysis [45]. However, in distinction to fibrin, FGF-2 bound to heparin sulfate proteoglycans in extracellular matrix is inactive and must be solubilized to recover activity. Fibrinogen may serve as a matrix binding site for FGF-2 as it is found in both normal and atherosclerotic arterial walls [46,47]. Fibrin formation also occurs following hemostatic activation at sites of vessel injury or inflammation, and it both contributes to the hemostatic plug and also provides a provisional matrix to support local cell responses. Fibrin(ogen)-bound FGF-2 may serve to both localize activity and prevent proteolytic inactivation and also concurrently activate both growth factor and integrin receptors. Mice lacking FGF-2 show delayed wound healing [48], and the lack of cofactor function of fibrinogen may also contribute to poor wound healing observed in fibrinogen-deficient mice [49].

The binding of FGF-2 to fibrinogen has implications regarding the distribution and actions of FGF-2 within the vasculature. FGF-2 is present normally in plasma at a concentration up to 0.6 pM, and elevated levels up to 6 pM can be found in patients after cardiopulmonary bypass [50], chronic liver disease [51], sarcoma [52], various carcinomas [53]. At normal plasma concentrations of fibrinogen (7 μM) and of FGF-2 (up to 6 pM) nearly all FGF-2 should be bound to fibrinogen considering the Kds in the nM range [32]. However, other FGF-2 binding proteins, α_2-macroglobulin [54] and soluble forms of FGF receptor [55], have also been identified in blood. The binding of FGF-2 to α_2-macroglobulin involves formation of covalent bonds and is slow, requiring up to 4 h to reach completion [54]. Three soluble truncated forms of the high-affinity cell receptor FGFR1 have also been identified in plasma as binding proteins for FGF-2 [55], but neither the plasma concentration nor binding affinities have been described. Further studies will be required to elucidate the distribution of FGF-2 binding to these plasma proteins and their role in influencing plasma half-life or FGF-2 activity.

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