Potentiation of Endothelial Cell Proliferation by Fibrinogen-bound Fibroblast Growth Factor-2*

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Endothelial cell growth is stimulated by fibroblast growth factor-2 (FGF-2), and both adhesion and proliferation are modulated by interactions with fibrinogen and fibrin. Previous evidence indicates that FGF-2 binds specifically and with high affinity to fibrinogen and fibrin, suggesting that their effects on endothelial cells may be coordinated. In this study, we have, therefore, investigated the ability of FGF-2 bound to fibrinogen and fibrin to stimulate proliferation of endothelial cells. Human umbilical vein endothelial cells were cultured in the presence of FGF-2 with or without fibrinogen, and proliferation was assessed by microscopic examination of cultures, incorporation of [3H]thymidine, and by cell counting. Cells cultured in the presence of both FGF-2 and fibrinogen proliferated more rapidly than those with FGF-2 alone and exhibited a decreased population doubling time. At concentrations of FGF-2 up to 150 ng/ml, there was greater endothelial cell proliferation in the presence of fibrinogen than in its absence with the most pronounced effect below 1 ng/ml. The maximum effect of fibrinogen was observed at a molar ratio of fibrinogen to FGF-2 of 2:1, corresponding to the maximum molar binding ratio. Endothelial cells proliferated when plated on fibrin or surface-immobilized fibrinogen with FGF-2, indicating that FGF-2 bound to surface-associated fibrinogen retained activity. We conclude that fibrinogen- or fibrin-bound FGF-2 is able to support endothelial cell proliferation and that fibrinogen potentiates the proliferative capacity of FGF-2.

Endothelial cells normally have a low rate of proliferation in the adult with a life span of 100–10,000 days (1), but the endothelium retains its capacity for proliferation, which occurs physiologically in the corpus luteum and uterus and also during wound healing. Endothelial cell proliferation, differentiation, and migration are also needed for angiogenesis, an important process in many pathologic conditions including tumor growth, diabetic retinopathy, inflammation, and ischemic thrombotic diseases. Polypeptide growth factors play an important role in angiogenesis, and several stimulatory and inhibitory molecules have been identified (2, 3) including the new amino terminus of the fibrin chain has been identified (30) and recently shown to be the new amino terminus of the fibrin chain has been identified (30) and recently shown to be

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The abbreviations used are: FGF-2, fibroblast growth factor-2; PBS, phosphate-buffered saline; ECGS, endothelial cell growth supplement; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase in situ labeling.

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hemin, and the proliferative activity of FGF-2 is enhanced by binding to heparin (33–35). In this study we have investigated the ability of fibrinogen- and fibrin-associated FGF-2 to stimulate proliferation of endothelial cells in vitro. The results indicate that FGF-2 associated with surface-immobilized fibrinogen or fibrin retains its mitogenic activity, and that the endothelial cell proliferative response to FGF-2 is potentiated by fibrinogen.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Primary endothelial cells were obtained from human umbilical veins as described previously (36), seeded on 0.2% w/v gelatin-coated 25-cm² tissue culture flasks and cultured in McCoy’s 5A medium (Flow Laboratories, McLean, VA) containing 20% fetal bovine serum (FBS), 50 μg/ml endothelial cell growth supplement (ECGS) (Collaborative Research, Inc., Bedford, MA) and 100 μg/ml heparin (Sigma) until they reached confluence, typically within 4–5 days. The cells were passaged up to two times before use and then placed in suspension by trypan blue staining, whereas apoptotic nuclei, which have incorporated the digoxigenin labeled nucleotides, exhibit green fluorescence. Normal nuclei exhibit orange fluorescence due to propidium iodide staining, whereas apoptotic nuclei, which have incorporated the digoxigenin labeled nucleotides, exhibit green fluorescence.

**Determination of Population Doubling Time—**Endothelial cells were grown in McCoy’s 5A medium containing 20% FBS, 25 ng/ml FGF-2, and 1 unit/ml heparin and allowed to adhere for 6 h. The medium was then removed, and the cells were washed twice with serum-free McCoy’s 5A medium. Serum-free medium was then added containing 1% Nutridoma® (Roche Molecular Biochemicals), 25 ng/ml human recombinant FGF-2 (R&D Systems, Minneapolis, MN) and 1 μCi/ml [3H]thymidine (NEN Life Science Products) in the presence or absence of 10 μg/ml fibrinogen. After incubation at 37 °C for 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, and precipitates were collected on a filter using a manifold. Filters were washed twice with ice-cold 5% trichloroacetic acid, 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.

**Fibrinogen and Fibrin Preparation—**Human fibrinogen was obtained from American Diagnostica (Greenwich, CT), and copurifying fibronectin was removed by gelatin-Sepharose chromatography (38). Residual fibronectin remaining was further depleted by immunoaffinity chromatography as described elsewhere (39). The fibronectin concentration was determined by enzyme-linked immunosorbent assay (American Diagnostica) and represented less than 0.02% of the total protein. Cell culture wells were coated by incubation for 1 h at 25 °C with 0.4 ml of 10 μg/ml fibrinogen in McCoy’s 5A medium. Excess fibrinogen was aspirated, and the wells were washed twice with McCoy’s 5A medium before the cells were plated. Fibrin-coated wells were prepared using 1 mg/ml fibrinogen in McCoy’s 5A medium to which 1 unit/ml thrombin (Calbiochem-Novabiochem Corp.) was added, mixed, and rapidly pipetted into 24-well cell culture plates. The solution was aspirated after 45 s and before polymerization, leaving a thin coating of fibrin on the surface.

**Measurement of Apoptotic Nuclei by Terminal Deoxynucleotidyl Transferase (TUNEL) Assay—**Endothelial cells cultured on Tissue-Tek® (Sakura, Torrance, CA) were double-labeled using TUNEL assay (38) to detect apoptosis. Briefly, approximately 2 × 10⁴ endothelial cells suspended in McCoy’s 5A medium. Excess fibrinogen was removed by gelatin-Sepharose chromatography (38). The fibronectin concentration was determined by enzyme-linked immunosorbent assay (American Diagnostica) and represented less than 0.02% of the total protein. Cell culture wells were coated by incubation for 1 h at 25 °C with 0.4 ml of 10 μg/ml fibrinogen in McCoy’s 5A medium. Excess fibrinogen was aspirated, and the wells were washed twice with McCoy’s 5A medium before the cells were plated. Fibrin-coated wells were prepared using 1 mg/ml fibrinogen in McCoy’s 5A medium to which 1 unit/ml thrombin (Calbiochem-Novabiochem Corp.) was added, mixed, and rapidly pipetted into 24-well cell culture plates. The solution was aspirated after 45 s and before polymerization, leaving a thin coating of fibrin on the surface.

**RESULTS**

FGF-2 is needed to support endothelial cell growth and survival in culture. To determine whether it retains this activity when bound to fibrinogen, cells were cultured in medium containing 1.5–3 nM FGF-2 in the presence or absence of 30 nM fibrinogen. Because our previous studies demonstrated that FGF-2 binds to fibrinogen with an apparent kᵦ of 1.3 nM, the concentration of free FGF-2 under these conditions would be less than 0.01 nM and insufficient to support cell growth. Cells proliferated well in the presence of fibrinogen and FGF-2 and appeared normal microscopically (Fig. 1A). Apoptosis, which is known to be a consequence of growth factor deprivation, did not occur when cells were cultured with fibrinogen and FGF-2 (Fig. 1B) but was observed with fibrinogen but no FGF-2 (Fig. 1C).
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These initial microscopic observations indicated that the presence of fibrinogen did not block the biologic activity of FGF-2. The effect of fibrinogen on FGF-2-induced proliferation of endothelial cells was further examined by cell counting and determination of population doubling times. Cells were grown in the presence or FGF-2 with or without fibrinogen in the medium. Hirudin was included to inhibit the low levels of thrombin present in fetal bovine serum and prevent conversion of fibrinogen to fibrin. Population doubling times were calculated using endothelial cells between passages 2 and 8, which were cultured using 25 ng/ml FGF-2 and 10 μg/ml fibrinogen (gray bars), with 25 ng/ml FGF-2, and no fibrinogen (open bars), with 25 ng/ml FGF-2 and 10 μg/ml fibrinogen (hatched bars) or 10 μg/ml fibrinogen and no FGF-2 (black bars).

The effect of fibrinogen on FGF-2-mediated endothelial cell proliferation was also evaluated by [3H]thymidine incorporation (Fig. 2). In the absence of FGF-2, there was no increase in [3H]thymidine uptake between 6 and 48 h, indicating little or no cell proliferation. Also, little incorporation resulted from the addition of 10 μg/ml fibrinogen to the medium in the absence of FGF-2. As expected, FGF-2 alone stimulated proliferation, and [3H]thymidine incorporation was increased over control at all times points. Addition of fibrinogen potentiated proliferation mediated by FGF-2, with the greatest effect evident at 24 h (p < 0.04). There was little additional proliferation at later times, reflecting contact inhibition (not shown). Microscopic examination confirmed slower growth in the absence of fibrinogen, and a longer time was needed to reach confluence.

Cell proliferation was dependent on FGF-2 concentration in the presence and absence of fibrinogen (Fig. 3), with a maximum 6.4-fold increase in [3H]thymidine incorporation at 25 ng/ml in the absence of fibrinogen. There was, however, greater proliferation at all concentrations of FGF-2 in the presence of fibrinogen than in its absence. The effect of fibrinogen was particularly evident at FGF-2 concentrations below 1 ng/ml (Fig. 3, inset). For example, at 0.1 ng/ml FGF-2, there was 1.2 ± 0.5-fold increase over baseline in the absence of fibrinogen but 2.8 ± 0.5-fold in its presence (p < 0.001). The FGF-2 concentration dependence of endothelial cell proliferation was more complex in the presence of fibrinogen than in its absence. Maximum proliferation in the presence of both fibrinogen and FGF-2 occurred at 25 ng/ml, with a 13.7 ± 2.7-fold increase in comparison with 5.5 ± 0.2-fold in the absence of fibrinogen at the same FGF-2 concentration (p < 0.03). In the presence of fibrinogen, proliferation decreased at FGF-2 concentrations over 25 ng/ml, declining to 8.8 ± 0.6-fold over baseline at 100 ng/ml and 8.1 ± 0.5-fold at 150 ng/ml. At both latter concentrations, however, the [3H]thymidine incorporation remained higher than in the absence of fibrinogen.

The capacity to potentiate FGF-2 stimulated cell proliferation was also characterized over a range of fibrinogen concentrations (Fig. 4). An increase in cell proliferation was observed at 0.25 μg/ml (0.75 nM), and there was progressive enhancement of activity to a maximum of 2.9-fold over baseline at 5 μg/ml (15 nM), representing a molar ratio of fibrinogen to FGF-2 of 2:1. At higher concentrations of fibrinogen, no increased effect on cell proliferation was observed.

Following tissue injury, thrombin converts fibrinogen to fibrin, an insoluble polymer that forms the initial matrix required for cell adhesion and wound healing. To determine whether FGF-2 was active when bound to fibrinogen or fibrin presented as an adhesive substrate, we prepared surfaces coated with either fibrin or fibrinogen with or without added FGF-2. Endothelial cells were cultured on these surfaces and viewed microscopically. Cells grown on surfaces of fibrinogen (Fig. 5A) or fibrin (Fig. 5B) in the absence of FGF-2 were consistently sparse, but incorporation of FGF-2 into the matrix
polyacrylamide gel electrophoresis. The migration pattern of
0.04 for both).

determined endothelial cell proliferation.
Endothelial cells were plated on
FBS, 50
mM ECGS, and 100 µg/ml heparin and allowed to adhere for
6 h. The medium was then removed. Cells were washed twice, and then
serum-free medium was added containing 1% Nutridoma, and 25 ng/ml
FGF-2, and 1 µCi/ml 
[3H]thymidine in the presence of different concentra-
tions of fibrinogen. The molar ratios of FGF-2 to fibrinogen varied
from 0.1–2. After 24 h of incubation, nonadherent cells were removed,
and isotope incorporated into DNA was extracted with trichloroacetic
acid. Precipitates were collected by vacuum filtration, and incorporated
isotope was measured by scintillation counting.

resulted in marked proliferation (Fig. 5, C and D). Gelatin was
used as an alternative adhesive substrate, and wells coated
with a solution of gelatin to which FGF-2 had been added did
not adequately support proliferation (Fig. 5E). In control wells,
however, endothelial cells grew well on a coating of gelatin if
FGF-2 was included in soluble form in the culture medium (Fig.
5F). As quantitated by 
[3H]thymidine incorporation, prolifera-
tion was minimal on a surface of either fibrinogen or fibrin in
the absence of FGF-2 (Fig. 6), but was significantly enhanced
with FGF-2 immobilized with either fibrinogen or fibrin (p <
0.04 for both).

Samples of culture medium containing fibrinogen were col-
collected following the 24 h incubation and analyzed by SDS-
polyacrylamide gel electrophoresis. The migration pattern of
Aα, Bβ, and γ chains was unchanged and showed no fibrin-
oproteptide A or fibrinopeptide B cleavage, indicating that there was
no proteolytic degradation or conversion of fibrinogen into
fibrin (data not shown). To determine whether other adhesive
glycoproteins also stimulated cell proliferation with FGF-2,
endothelial cells were also cultured in the presence of vitronec-
tin (10 µg/ml) or fibronectin (10 µg/ml). Neither fibronectin nor
vitronectin increased proliferation significantly, indicating the
specificity of fibrinogen in enhancing the effect of FGF-2.

DISCUSSION

The findings presented demonstrate that fibrin- or fibrino-
gen-bound FGF-2 retains biological activity. Cells cultured in
medium containing 1.5 nm FGF-2 and 30 nm fibrinogen sup-
ported proliferation and did not undergo apoptosis, which is
known to occur under conditions of growth factor deprivation
(8). At the concentrations of fibrinogen and FGF-2 used in these
experiments, the amount of free FGF-2 would be insufficient to
support cell growth. FGF-2 associated with surface-immobi-

dized fibrinogen or fibrin was also active as indicated by its
ability to support growth (Fig. 5). Cell proliferation was quan-
titated by 
[3H]thymidine incorporation, determination of pop-
ulation doubling times, and cell counting. The findings with
each of these methods indicated that the proliferative potential
of FGF-2 is enhanced in the presence of fibrinogen. The popu-
lation doubling time was shorter with fibrinogen, the prolifer-
ative rate was greater (Fig. 2), and the cells responded to a
lower concentration of FGF-2 when presented in combination
with fibrinogen (Fig. 3). At all FGF-2 concentrations, fibrinogen
increased its proliferative capacity (Fig. 3), and the maximum
effect was observed at a molar ratio of fibrinogen to FGF-2 of
2:1 (Fig. 4), corresponding to the maximum molar binding ratio
(32).

Fibrinogen is an adhesive substrate for endothelial cells, but
it is unlikely that the enhanced proliferation observed was due
to an effect of fibrinogen on adhesive properties. Cells were
fully spread before FGF-2 exposure, and no change in cell
spreading was observed during incubation with fibrinogen. No
significant increase in FGF-2-induced cell proliferation was
observed with fibronectin or vitronectin, indicating the fibrin-
ogen effect was specific. Also, enhanced cell proliferation was
observed with fibrinogen even in medium containing 20% fetal
bovine serum, which is rich in adhesive proteins, confirming
the specificity of the enhancement by fibrinogen. Proliferation
was evaluated using several methods because of the experi-
mental limitations of each. Cultures were examined microscopi-
cally to evaluate morphologic characteristics including spreading
and apoptosis in addition to proliferation. 
[3H]Thymidine
incorporation was measured as an overall index of DNA syn-
thesis, recognizing that proliferation may be underestimated
with prolonged exposures. Population doubling time was deter-
mined as a direct measure of cell proliferation during prolonged
exposure to FGF-2 and fibrinogen with multiple cell passages.
Each of these methods indicated significant enhancement of
FGF-2 proliferative capacity in the presence of fibrinogen.

The mechanism by which fibrinogen potentiates FGF-2 ac-
tivity is not known but may involve receptor clustering or
coordination of cell signaling. Because fibrinogen is a dimeric
molecule, binding of two or more FGF-2 molecules could in-
crease cell activation through receptor dimerization analogous
to that observed with heparin (41). Also, binding of a complex
of fibrinogen and FGF-2 could result in co-localization of integrin
and FGF receptors at the focal adhesion complex, contributing
to signal integration (42). Fibrinogen may protect FGF-2 from
inactivation by serum or cell-associated proteases, thereby pro-
longing and increasing its activity. Such protection from pro-
teolytic degradation has been observed for FGF-2 bound to
extracellular matrix (43, 44). Additionally, a recent report in-
dicates that FGF-2 can bind to α6β1 (45), an endothelial cell
integrin receptor that also binds fibrinogen, suggesting that
the adhesive and proliferative activities of FGF-2 and fibrino-
ogen may be coordinated through a single receptor.

Endothelial cell responses to injury and angiogenesis are
dependent on both growth factor stimulation and interactions
with matrix components. The importance of endothelial cell-
matrix interactions in angiogenesis is evident from the binding
of FGF-2 to extracellular matrix heparan sulfate proteogly-
cans. Although of lower affinity than the binding to specific
tyrosine kinase receptors, the association with heparan sul-
fates is physiologically important in protecting FGFs from pro-
teolytic degradation (43, 44, 46) and by providing a local reser-
voir of growth factor that can be released by enzymes that
degrade proteoglycans (47, 48). Additionally, heparan sulfates
increase the binding affinity of FGFs for specific receptors and
facilitate presentation to transmembrane signaling receptors
(41). The mechanisms by which heparan sulfate proteoglycans
modulate FGF function remain under investigation, but they
may act to reduce the dimensionality of ligand diffusion to a
plane from three dimensions (48). Endothelial cells are ex-
posed to fibrin both pathologically and in response to vessel injury
where fibrin forms the initial matrix necessary for cell organi-
ization and healing. As such, fibrin could play a role similar to
that of the extracellular matrix in binding FGF-2, which both
localizes and prolongs its action.

FIG. 4. Fibrinogen concentration dependence of FGF-2-mediated endothelial cell proliferation. Endothelial cells were plated on
gelatin-coated wells in McCoy’s 5A medium supplemented with 20% FBS, 50 µg/ml ECGS, and 100 µg/ml heparin and allowed to adhere for
6 h. The medium was then removed. Cells were washed twice, and then
serum-free medium was added containing 1% Nutridoma, and 25 ng/ml
FGF-2, and 1 µCi/ml 
[3H]thymidine in the presence of different concentra-
tions of fibrinogen. The molar ratios of FGF-2 to fibrinogen varied
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Endothelial cells cultured on fibrinogen or fibrin in the presence or absence of FGF-2. Culture wells containing coverslips were coated with fibrinogen (A and C) or fibrin (B and D) with no FGF-2 (A and B) or with 25 ng/ml FGF-2 (C and D). In control experiments, cells were plated on tissue culture wells coated with gelatin and 25 ng/ml FGF-2 (E) or gelatin alone (F). The cells were cultured in McCoy's medium containing no FGF-2 (A–E) or containing 25 ng/ml FGF-2 (F). After 24 h, the cells were fixed, permeabilized in 0.5% Triton X-100, washed with PBS twice, stained with propidium iodide, and the coverslips were viewed with a fluorescein microscope. Bar = 100 μm.

The potential to manipulate angiogenesis therapeutically is now being realized in initial clinical trials, and strategies to either inhibit or stimulate new vessel growth appear promising (49, 50). The association of FGF-2 with fibrin(ogen) is relevant now being realized in initial clinical trials, and strategies to either inhibit or stimulate new vessel growth appear promising (49, 50). The association of FGF-2 with fibrin(ogen) is relevant

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