# Modulation of vascular cell growth kinetics by local cytokine delivery from fibrin glue suspensions

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***Purpose:*** **Fibrin glue (FG) has been used as a delivery system for bioactive agents on grafts and angioplasty sites. Reports from two different institutions suggest that heparin concentrations of 500 U/mL in FG inhibit smooth muscle cell (SMC) proliferation, but do not effect endothelial cell (EC) proliferation. The purposes of this study were to (1) quantify the diffusive release of fibroblast growth factor-1 (FGF-1) and heparin from FG; (2) determine the effect of heparin and FGF-1 on SMC proliferation when the cells are immediately plated on the FG; and (3) by means of the diffusive release data, design a new in vitro model that may differentiate the effect of FG-incorporated FGF-1 and heparin, rather than the released, solubilized components of these two factors, on SMC and EC proliferation.**

***Methods:*** **$^{125}$I-FGF-1 or $^{3}$H-heparin release from FG into the overlying media was measured serially in a 96-hour period, either with or without cells. SMCs were immediately plated on FG containing various concentrations of FGF-1 and heparin. SMCs or ECs were plated on identical groups of FG containing FGF-1 and heparin 24 hours after the FG was made to exclude the effect on cell growth of the initial release of FGF-1 into the media.**

***Results:*** **In the first 24 hours, 70% ± 1% of the FGF-1 and 59% ± 2% of the heparin in the FG was released into the overlying media, with minimal release occurring thereafter. The cell type or absence of cells did not affect release, but there was five times more FGF-1 and four times more heparin in the media at 72 hours for the immediate plating versus the delayed plating because of a diffusive release primarily in the first 24 hours. A heparin concentration of 500 U/mL inhibited SMC proliferation, as compared with 5 U/mL heparin, only when immediate plating of SMCs was used. Comparing immediate versus delayed SMC plating, at equivalent FGF-1 and heparin doses, immediate plating induced greater proliferation than delayed plating; this was likely caused by the higher soluble FGF-1 concentration. Heparin doses as high as 500 U/mL had little effect on SMC proliferation. In contrast, ECs died with delayed plating on FG containing 500 U/mL heparin, and their growth was inhibited at 50 U/mL heparin, as compared with 5 U/mL heparin.**

***Conclusion:*** **The differences in SMC proliferation when comparing immediate versus delayed plating are likely caused by diffusive release of heparin and FGF-1 into the media. Our ongoing work uses an optimized in vitro FG system that minimizes the effects of soluble factors. This is an important distinction, because the cytokines that are released in vivo will be removed by blood flow and, thus, may not exert an effect unless they are contained within the FG. (J Vasc Surg 1999;29:852-62.)**

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Supported by grants from the National Institutes of Health (R01 HL41272) and the Department of Veterans' Affairs.

Presented at the Twenty-second Annual Meeting of the Midwestern Vascular Surgical Society, Detroit, Mich, Sep 25-26, 1998. Winner of the Charles C. Gutherie Award for Outstanding Research in Vascular Surgery.

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0741-5214/99/$8.00 + 0 **24/6/97974**

The treatment of arterial occlusive disease leads to 550,000 vascular bypass grafting procedures and 500,000 carotid endarterectomies annually in the United States.[1,2] These procedures damage the endothelial lining of the blood vessels, often leading to thrombosis and intimal hyperplasia. Clinically, endothelial cell (EC) resurfacing of vascular grafts or of long segments of de-endothelialized arteries after endarterectomy or angioplasty does not occur. This has led many researchers to try and develop ways to replace the damaged EC lining.

One of these strategies involves fibrin glue (FG). FG has been used experimentally as an extended delivery system for cytokines on vascular grafts and angioplasty sites. Cytokines and heparin can be mixed with fibrinogen before the addition of thrombin. Thrombin cleaves the fibrinogen into fibrin, and a matrix is created that contains the cytokines and heparin. We believe that FG acts as a passive reservoir for the cytokines, but formal binding studies have not been performed. The body's fibrinolytic system slowly degrades the matrix, possibly releasing the cytokines.

Our group has previously reported a FG system containing fibroblast growth factor-1 (FGF-1), also known as acidic FGF, and heparin used on grafts implanted into the aortoiliac and thoracoabdominal aortic positions in dogs.[3,4] Expanded polytetrafluoroethylene (ePTFE) grafts were treated with FG containing 1 ng/cm$^2$ FGF-1 and 20 μg/cm$^2$ heparin. Control grafts were either untreated or treated with FG containing heparin but not FGF-1. The grafts treated with FGF-1/heparin/FG developed a significantly greater luminal cell mitotic index and complete EC coverage by 4 weeks, which was never seen in the explants of the control group of the canine aortoiliac grafts.[3] Similar results were reported with thoracoabdominal ePTFE-treated grafts in the canine model studied after 20 weeks.[4] Enhanced endothelial coverage of the graft was seen; however, inner capsule thickness was significantly greater in the FGF-1/heparin/FG group (139 ± 16 μm), as compared with either untreated grafts (67 ± 18 μm) or the FG-plus-heparin group (93 ± 9 μm). FGF-1/heparin/FG–treated grafts also had an increased cross-sectional mitotic index at 30 days, as measured by means of tritiated thymidine incorporation, which returned to baseline levels at the 20-week observation point.

FGF-1 stimulates both EC and smooth muscle cell (SMC) proliferation. Concern about intimal hyperplasia in the canine grafts receiving FGF-1 and FG led to a series of studies designed to optimize the FGF-1 and heparin ratios in vitro.[5] This optimization was defined as maximizing EC proliferation while minimizing SMC growth that may lead to intimal hyperplasia. We attempted to achieve this by manipulating the relative concentrations of heparin in the FG. Heparin, a known inhibitor of SMC proliferation, was added to the FG in increasing concentrations to determine the effect on EC and SMC proliferation. Human umbilical vein endothelial cells (HUVECs) or canine carotid artery SMCs were grown on FG containing 10 ng/mL FGF-1 and heparin concentrations of 0, 5, 50, or 500 U/mL, and proliferation was measured with tritiated thymidine incorporation. FG containing 10 ng/mL FGF-1 but no heparin did not induce EC or SMC proliferation higher than control levels (FG with no additives). This is presumably caused by thrombin inactivation of FGF-1. The addition of heparin to the FG protects the FGF-1 from thrombin inactivation. HUVEC proliferation was not affected by the heparin concentrations of 5, 50, or 500 U/mL, but canine carotid SMC proliferation was significantly inhibited by the addition of 500 U/mL heparin. Similar levels of SMC inhibition were also seen at a heparin concentration of 250 U/mL. These in vitro observations led us to conclude that the "optimal" FG to induce EC, but not SMC, proliferation for potential in vivo use would contain 10 ng/mL FGF-1 and a heparin concentration of 250 U/mL or higher.

This in vitro data[5] led us to use FG with 10 ng/mL FGF-1 and 250 U/mL heparin in a canine balloon-injury carotid model.[6] We measured surface re-endothelialization and were disappointed to find no significant difference between the FG-treated arteries and the negative controls that did not receive FG treatment. The addition of cultured, autologous, canine jugular vein endothelial cells to the FG/FGF-1/heparin mixture did result in a significant increase in surface reendothelialization than did the FG treatment alone.

Another group, Weatherford et al,[7] reported the in vitro optimization of vascular endothelial growth factor (VEGF) and heparin in FG. VEGF is a specific EC mitogen that does not induce SMC proliferation and thus would theoretically not have the disadvantage of increasing intimal hyperplasia. By using human aortic ECs and SMCs grown on FG containing various concentrations of VEGF and heparin, they also found that human EC proliferation was not affected by heparin concentration and that SMC proliferation was significantly inhibited by heparin in a dose-dependent fashion in the FG. They conclud-

ed that the optimal FG contained 10 ng/mL VEGF and 50 U/mL heparin.

These two in vitro studies[5,7] plated ECs and SMCs on the FG immediately after polymerization. Cytokines and heparin are released by diffusion from FG, and the soluble, released substances in the overlying media may affect cellular proliferation. In vivo, released substances would be carried away by blood flow and thus would not exert a local effect. In addition, species differences may exist in tissue culture cell lines. We believed that these cytokine-release issues may explain the disappointing results seen in our canine balloon-injured carotid artery model,[6] which used the in vitro optimized FG formulation.[5] We decided to study the release of FGF-1 and heparin in our in vitro model to determine whether the released cytokines and heparin may account for the SMC and EC proliferation effects demonstrated in our previous paper.[5] We hoped to reexamine our in vitro model and perhaps change it to more accurately reflect in vivo conditions. Therefore, we set out to: (1) quantify the diffusive release of FGF-1 and heparin from FG; (2) determine the effect of heparin and FGF-1 on SMC proliferation when the cells are immediately plated on the FG; and (3) by using the diffusive release data of FGF-1 and heparin from FG, design a new in vitro model with delayed plating of ECs and SMCs that may differentiate the effect of FG-incorporated FGF-1 and heparin, rather than the released, solubilized components of these two factors, on SMC and EC proliferation.

## METHODS

Animal care complied with "The Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) and the "Principles of Laboratory Animal Care" (National Institutes of Health publication no. 85-23, revised 1985).

**Endothelial and smooth muscle cell harvest.** Adult, mongrel dogs were anesthetized with thiopental sodium (Abbott Laboratories, Morris Plains, NJ), intubated, and ventilated. Anesthesia was maintained with nitrous oxide and halothane. Bilateral neck incisions were made. For ECs, bilateral external jugular veins were removed, inverted, and processed per our previously reported protocol.[6] In brief, the veins were sequentially placed into 0.53 mmol/L 0.05% trypsin/ethylenediamine tetra-acetic acid (EDTA; Gibco, Grand Island, NY) and 100 U/mL collagenase (Gibco) at 37°C for 10 minutes each. After discarding the veins, the trypsin and collagenase solutions were centrifuged at 1000 rpm for 10 minutes. The supernatants were discarded, and the cells were suspended in 5 mL EC growth media (M-199 [Gibco] supplemented with 10% fetal bovine serum [FBS; Hyclone, Logan, Utah], 5 ng/mL FGF-1 [American Red Cross, Rockville, Md], 5 U/mL bovine lung heparin [Pharmacia & Upjohn, Kalamazoo, Mich], 100 U/mL penicillin, and 100 μg streptomycin [Gibco]). The ECs were plated onto a fibronectin (American Red Cross) coated T-25 culture flask (2.5 μg/cm$^2$) and incubated at 37°C in a 5% humidified $CO_2$ chamber. The EC growth media was changed every 2 to 3 days, and confluent cells were passaged with trypsin-EDTA. EC identity was confirmed by means of dual staining with factor VIII (Dako, Carpenteria, Calif) and α-actin (Sigma Chemical, St. Louis, Mo) antibody immunofluorescent staining. Only EC cultures exhibiting 95% or more positive factor VIII staining and 5% or less positive α-actin staining were used for the proliferation assays. ECs were used within passages 1 through 4.

SMCs were obtained from canine carotid arteries with a previously published[5] explant technique. In brief, the carotid arteries were opened longitudinally, and the intima and adventitia were removed by scraping and dissecting with a scalpel. The media was minced and placed into SMC growth media (Dulbecco's modified Eagle's media [DMEM] supplemented with 10% FBS [Hyclone], 10 mmol/L L-nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 mmol/L L-sodium pyruvate, and 50 μg/mL gentamicin [all from Gibco]). Primary SMCs migrating from the explants were used in all experiments. SMC identity was confirmed by means of immunofluorescent staining with α-actin antibody (Sigma Chemical), and only cultures exhibiting 95% or more positive staining were used in the proliferation assays.

**Fibrin glue plate preparation.** FG was made by reconstituting lyophilized human fibrinogen (American Red Cross) prepared from donor-pooled plasma with 0.9% saline. For modified FG, heparin and FGF-1 were also added to obtain a solution with a final concentration of 32 mg/mL fibrinogen, FGF-1 at concentrations of 0, 10, 100, or 1000 ng/mL, and heparin at concentrations of 0, 5, 50, or 500 U/mL. Lyophilized human thrombin (American Red Cross) was reconstituted with 0.9% saline and added to all solutions to make a final concentration of 0.32 U/mL. A solution containing fibrinogen and thrombin only was made and used as the negative control. Before polymerization, the solutions were pipetted in 30 μL aliquots into 96-

well polystyrene plastic plates (Becton Dickinson, Lincoln Park, NJ) and allowed to completely polymerize, about 1 hour, before placing cells, culture media, or both into the wells.

**Release of radioactive fibroblast growth factor-1 and heparin from fibrin glue.** FG was made as described, except $^{125}$I-FGF-1 (American Red Cross) or $^{3}$H-heparin (NEN Life Sciences Products, Boston, Mass) was added to the FG. The $^{125}$I-FGF-1 was found to be more than 99% pure by means of sodium dodecyl sulfate/polyacrylamide gel electrophoresis and high-pressure liquid chromatography. An $^{125}$I-FGF-1 concentration of 500 ng/mL was chosen, because the specific activity of the $^{125}$I-FGF-1 would allow detection of as low as 1% release of FGF-1 into the media. A 500 ng/mL concentration of $^{125}$I-FGF-1 and heparin at a concentration of 5 or 500 U/mL were placed in the FG. After polymerization was complete, 200 μL of the EC or SMC media, which were used in the proliferation assays, was added to the wells. The media was removed at the specified time and counted for radioactivity (1,4, 8, 24, 48, 72, and 96 hours), and there were 4 wells per time point.

To measure heparin release, $^{3}$H-heparin was placed in FG at a dose of 5 or 500 U/mL. Either 10 or 1000 ng/mL FGF-1 was also placed in the FG with the 5 U/mL heparin to determine whether the FGF-1 concentration affected heparin release. Media was assayed at 4-, 24-, 48-, and 96-hour points, and there were three replicate wells per time point.

In addition, 30 μL of each FG solution was pipetted into Ready Safe Scintillation Fluid (Beckman Instruments, Fullerton, Calif) for $^{3}$H-heparin or into tubes for gamma counting for $^{125}$I-FGF-1 in replicates of 3 or 4, respectively. The average of the 30 μL FG samples represented the total counts. Percent release of radio-labeled FGF-1 or heparin was calculated by dividing the media counts from each well by the average of the total FG counts and multiplying by 100%. Data is expressed as the mean percentage released plus or minus standard deviation for each time point. $^{125}$I-FGF-1 or $^{3}$H-heparin was not corrected for decay caused by the short duration of the experiments, as compared with the half-life of the isotopes (60 days and 12.5 years, respectively).

For $^{125}$I-FGF-1, after counting the radioactivity within the media samples, precipitation experiments were performed to discriminate protein-bound versus free $^{125}$I. To each 200 μL media sample, 200 μL 0.1% bovine serum albumin (Sigma Chemical) and 100 μL 50% trichloroacetic acid (Sigma Chemical) were added. The samples were incubated on ice for 30 minutes, and 1000 μL 10% trichloroacetic acid was added. The samples were centrifuged at 4°C for 8 minutes at 5000 rpm, and the supernatant was removed. The precipitate, which contained the $^{125}$I that was bound to FGF-1, was counted and the percentage bound was calculated by dividing the precipitate counts by the media counts and multiplying by 100%. The data are expressed as the mean percentage bound plus or minus standard deviation.

To assess whether the presence of cells affected the release of substances from FG, ECs or SMCs were plated on the FG 1 hour after polymerization (immediate plating) or after removing and replacing media that had been in contact with the FG for 24 hours before cell plating (delayed plating). Cells were plated at 5000 cells per well and allowed to grow for 72 hours before removing the media for counting. Identical wells of FG had media only placed on them in either an immediate or a delayed fashion as controls.

**Endothelial cell and smooth muscle cell proliferation assays.** Confluent cells were trypsinized and plated on FG at 5000 cells/well in 200 μL of media. Preliminary studies used to optimize the FG assay revealed that using 5000 cells/well resulted in a consistent and easily measurable difference between the negative control and cytokine containing FG. ECs were plated in media devoid of FGF-1 and heparin (M-199, 10% FBS, penicillin, and streptomycin). SMCs were plated in SMC growth media (DMEM, 10% FBS, L-sodium pyruvate, L-nonessential amino acids, penicillin, streptomycin, and gentamicin). Media was placed on the FG for 24 hours and removed before plating cells to minimize the effect of soluble FGF-1 and heparin known to be released in the first 24 hours (delayed plating). Additional groups of SMCs were also plated 1 hour after the FG was made (immediate plating) to reproduce the inhibition on SMC proliferation by 500 U/mL heparin in the FG that was reported in the previously published papers.[5,7]

After plating, the cells were allowed to grow for 48 hours, then 1 μCi/well $^{3}$H-thymidine (NEN Life Sciences Products) was added, and the plates were processed 24 hours later. $^{3}$H-heparin, which was used in the release studies, was not used at any time during the proliferation assays. The plates were processed by removing the media and washing the wells with 0.9% saline. The cells were fixed in 100% methanol (Fischer Scientific, Fair Lawn, NJ) for 10 minutes, lysed with distilled water, and the DNA was

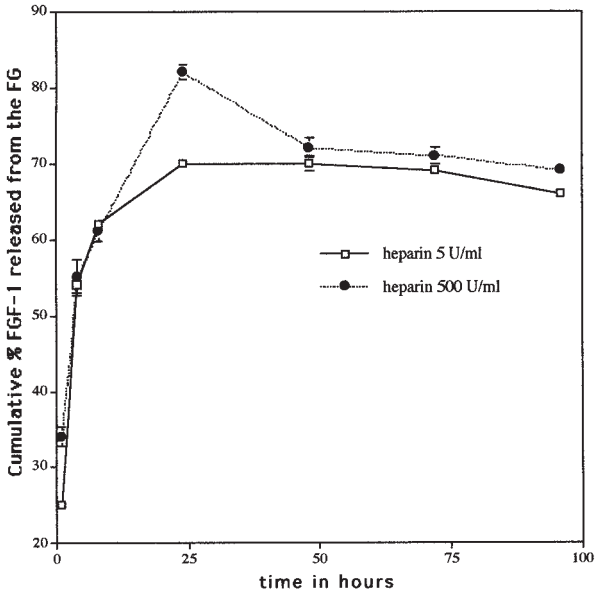


**Fig 1.** Release of $^{125}$I-fibroblast growth factor-1 (FGF-1) from fibrin glue (FG) into the media. The media was placed on FG containing 500 ng/mL $^{125}$I-FGF-1 with heparin at a concentration of 5 or 500 U/mL, and the release of $^{125}$I-FGF-1 was serially measured at 1, 4, 8, 24, 48, 72, and 96 hours. The data are expressed as mean ± standard deviation of percentage of FGF-1 in the media. N = 4 for each observation. Error bars not seen indicate standard deviation less than 1%.

precipitated with 5% trichloroacetic acid. The cell lysates were washed with distilled water, and the FG was solubilized by adding 100 μL of 0.3 mol/L NaOH (Sigma Chemical) and heating at 60°C for 35 minutes. This solution was placed into 10 mL scintillation fluid that contained 20 μL acetic acid (Fischer Scientific) to avoid opacification of the scintillation fluid with an accompanying dampening of the counts per minute (CPM).

During preliminary experiments, we found that the FG itself has a very high background that was not affected by the composition of the FG and exhibited very little variance. Therefore, every plate contained wells with FG but no cells, and the average of these wells was subtracted from the CPM of the test wells to eliminate the high FG background. Cells plated on FG with no additives were also placed on each plate as a negative control, and the data were normalized by dividing the CPM of each well by the average of the negative control wells and multiplying by 100%:

$$\%\ \text{of negative control} = ([\text{CPM-BKG}_{ave}]/[\text{negative control average CPM-BKG}_{ave}])100\%$$

The data are expressed as the mean percentage of the negative control plus or minus standard deviation. All proliferation experiments were performed in duplicate, and each treatment had 6 replicates in each experiment (n = 12 for the combined data). After normalizing the data from each experiment, the observations from the two experiments were combined to calculate the mean of the percentage of the negative control plus or minus standard deviation.

**Statistics.** Analysis of variance with Fischer's Least Square Differences was used as an adjunct test, and unpaired Student *t* tests were performed. The results for these tests were reported as significant at the $P < .05$ level.

## RESULTS

**Release of $^{125}$I-FGF-1 and $^{3}$H-heparin from fibrin glue.** The results of the release of FGF-1 and heparin from FG are indicative of a burst effect commonly seen with matrix types of release systems. As shown in Fig 1, 70% ± 1% of the $^{125}$I-FGF-1 in the FG containing 5 U/mL heparin was released into the overlying SMC media in the first 24 hours, with minimal release occurring thereafter (66% ± 1% at 96 hours for 5 U/mL heparin). The results were similar for both the 5 and 500 U/mL heparin concentrations, suggesting that diffusive release of FGF-1 from FG is independent of the tested heparin concentrations. The media used for the EC proliferation assays was also placed in wells containing FG with 500 ng/mL $^{125}$I-FGF-1 and 5 U/mL heparin, and these results were also similar to the release into the SMC media (EC media $^{125}$I-FGF-1 release at 24 hours, 65% ± 1%).

With this data, we wanted to minimize the concentration of released, soluble FGF-1 in the media in our in vitro assay. Noting that most of the FGF-1 release occurred within the first 24 hours, we decided to measure the amount of $^{125}$I-FGF-1 in the media with immediate versus delayed plating.

Both ECs and SMCs synthesize fibrinolytic substances that may potentially degrade the fibrin matrix and thus may alter the release of $^{125}$I-FGF-1. Therefore, we compared media only with media containing 5000 ECs or SMCs plated in an immediate or a 24-hour delayed fashion. By using the 24-hour delayed plating, we hoped to decrease the amount of soluble $^{125}$I-FGF-1 in the media. SMCs, ECs, or media only (no cells) were plated on FG containing 500 ng/mL $^{125}$I-FGF-1 with 5 U/mL heparin in an immediate and delayed (media changed 24 hours after FG polymerization) fashion for 72 hours to determine the percent release of

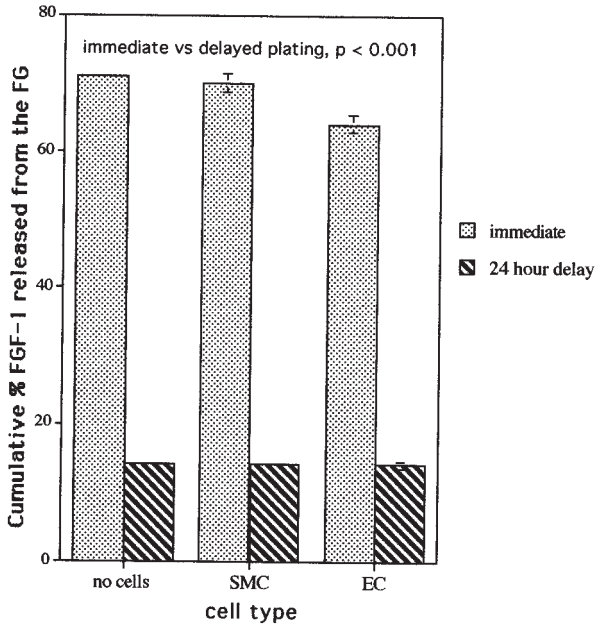


**Fig 2.** Effect of immediate versus delayed plating on release of $^{125}$I-fibroblast growth factor-1 (FGF-1) from fibrin glue (FG). Cells were plated on FG containing 500 ng/mL $^{125}$I-FGF-1 with 5 U/mL heparin in an immediate or in a 24-hour delayed fashion, and the release of $^{125}$I-FGF-1 into the media was measured 72 hours after the cells were plated. The data are expressed as mean ± standard deviation of percentage $^{125}$I-FGF-1 in the media. N = 4 for each observation. SMC, Smooth muscle; EC, endothelial cell.

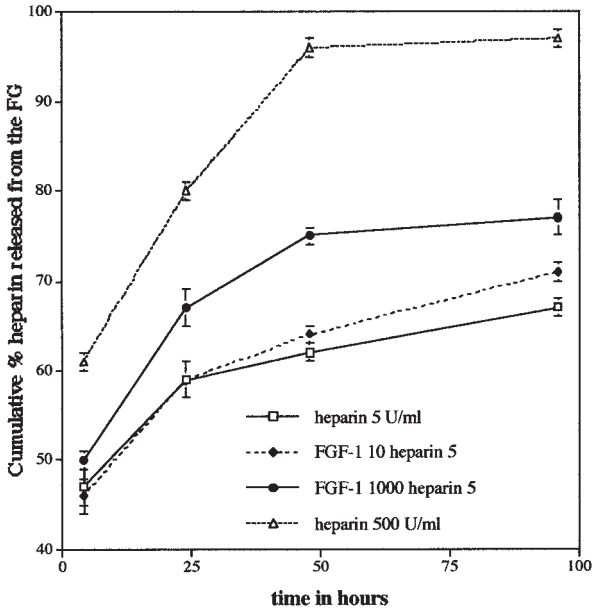


**Fig 3.** Release of $^{3}$H-heparin from fibrin glue (FG) into the media. Media was placed on FG containing 5 or 500 U/mL $^{3}$H-heparin, either alone or in combination with 10 or 1000 ng/mL fibroblast growth factor-1 with 5 U/mL $^{3}$H-heparin, and the release of $^{3}$H-heparin was serially measured at 4, 24, 48, and 96 hours. The data are expressed as mean ± standard deviation of percentage of heparin in the media. N = 3 for each observation.

FGF-1 into the media with the two methods of plating. The cell type or absence of cells did not affect FGF-1 release, but there was five times more FGF-1 in the media for the immediate versus delayed plating ($P < .001$; Fig 2).

After counting, all the medias underwent precipitation experiments to determine the percentage of $^{125}$I that was bound to the FGF-1. The mean percentage bound for the entire experiment was 93% ± 3%.

The results for 5 U/mL $^{3}$H-heparin release from FG were comparable with the $^{125}$I-FGF-1 diffusive release. At 24 hours, 59% ± 2% $^{3}$H-heparin was released, with minimal release thereafter (67% ± 1% at 96 hours). There was no significant difference in $^{3}$H-heparin release into EC or SMC media. The addition of FGF-1 at 10 or 1000 ng/mL concentrations had a minimal effect on the release of $^{3}$H-heparin (59% ± 2% and 67% ± 2% at 24 hours for FGF-1 at 10 and 1000 ng/mL concentrations, respectively; Fig 3).

There was a significantly higher percentage of $^{3}$H-heparin released at a concentration of 500 U/mL than at one of 5 U/mL ($P < .001$ for both the 24- and 96-hour time points). Release at the 500 U/mL concentration was 80% ± 1% at 24 hours and 97% ± 1% at 96 hours. This is thought to be potentially related to artifact. There were difficulties in measuring the CPM of the 500 U/mL $^{3}$H-heparin, because the CPM of these samples was initially too high to measure. Serial dilutions of these samples were performed until they could be counted. This may have led to differences in counting efficiency. This potential artifact may have especially affected the total counts measured in the 30 μL of FG that was initially placed into 10 mL of scintillation fluid (the denominator), thus leading to lower total counts and, therefore, a higher percent released.

SMCs or media only (no cells) were plated on FG containing 5 U/mL $^{3}$H-heparin in an immediate or a delayed fashion. The presence or absence of cells did not affect heparin release, but there was four times more heparin in the media for the immediate versus the delayed plating ($P < .001$; Fig 4).

**Endothelial cell and smooth muscle cell pro-**

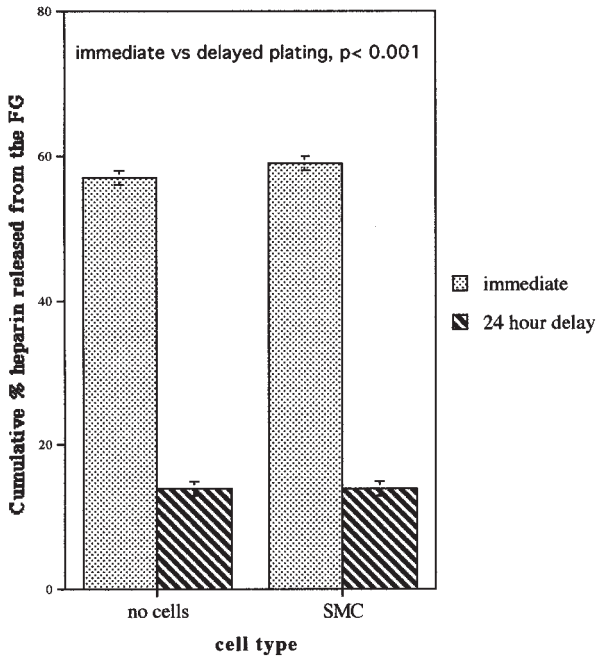


**Fig 4.** Effect of immediate versus delayed plating on release of $^{3}$H-heparin from fibrin glue (FG). Cells were plated on FG containing 5 U/mL $^{3}$H-heparin in an immediate or in a 24-hour delayed fashion, and the release of $^{3}$H-heparin into the media was measured 72 hours after the cells were plated. The data are expressed as mean ± standard deviation of percentage of $^{3}$H-heparin in the media. N = 3 for each observation.
SMC, Smooth muscle cell.

**liferation assays, delayed plating.** Delayed plating of ECs and SMCs was performed on FG to minimize the effect of the release of FGF-1 and heparin that occurs within the first 24 hours. This is an important point, because substances released from FG in vivo will be removed by blood flow and thus may not exert a local effect. Substances released from FG in vitro will be soluble in the overlying media and, if the media is not removed before cell plating, can thereby affect SMC and EC proliferation. The data for both EC and SMC proliferation assays are shown in Table I. We did not test FG with FGF-1 in the absence of heparin caused by our two previous studies,[5,8] which showed that FGF-1 alone in the FG did not induce any significantly greater proliferation than the negative control.

EC proliferation assays on FG containing FGF-1 concentrations of 0, 10, 100, or 1000 ng/mL and heparin concentrations of 0, 5, 50, or 500 U/mL revealed that the lowest tested FGF-1 concentration that produced a maximal proliferative effect occurred at 100 ng/mL FGF-1 and 5 U/mL heparin (all with 5 U/mL heparin; $P < .0001$ for 100 mg/mL FGF-1 versus 10 mg/mL FGF-1, and $P = .16$ for 100 ng/mL FGF-1 versus 1000 ng/mL FGF-1). As the heparin dose increased to 50 U/mL, EC proliferation decreased, and at 500 U/mL, canine jugular ECs died. Previous experiments have shown that FG that contains FGF-1, in the absence of heparin, does not induce EC or SMC proliferation higher than control levels, because the thrombin in the FG degrades the FGF-1, thereby inactivating it.[5,8]

Heparin doses as high as 500 U/mL had little effect on SMC proliferation when cells were plated in a delayed fashion on FG containing FGF-1. In the absence of FGF-1, there was a trend for decreased SMC proliferation with increasing heparin in the FG, but these differences were not statistically significant ($P = .065$ for 5 U/mL heparin versus 500 U/mL heparin). Similar to the ECs, FGF-1 at a concentration of 100 ng/mL gave a maximal proliferative effect (no significant difference between 100 ng/mL FGF-1 and 1000 ng/mL for the heparin 50 and 500 concentrations; $P < .001$ for 100 ng/mL FGF-1 versus 1000 ng/mL for the 5 U/mL heparin concentration; $P < .0001$ for 10 ng/mL FGF-1 versus 100 ng/mL for all three heparin concentrations).

**Smooth muscle cell proliferation assays, immediate plating.** As shown in Table II, heparin at a 500 U/mL concentration inhibited SMC proliferation, as compared with concentrations of 5 or 50 U/mL, when immediate plating was used ($P < .001$ for all three doses of FGF-1 when comparing the 5 U/mL heparin versus the 500 U/mL heparin concentration). Heparin at a 50 U/mL concentration inhibited SMC proliferation when 10 ng/mL FGF-1 was present, as compared with FG containing 10 ng/mL FGF-1 and 5 U/mL heparin ($P < .01$). As the FGF-1 dose increased to 100 and 1000 ng/mL, the FGF-1 overcame the effect of the heparin, and there was no significant difference in SMC proliferation at these two FGF-1 doses when comparing heparin at concentrations of 5 or 50 U/mL. The inhibition of SMC proliferation with increasing heparin dose is likely caused by the soluble, released heparin at 24 hours. By means of the radioactive heparin release data, the heparin concentration in the media would be approximately 45 U/mL, which is a high enough concentration to inhibit SMC proliferation.[8] Comparing immediate versus delayed plating at equivalent FGF-1 and heparin concentrations, immediate plating induced greater proliferation than delayed plating, likely because of the higher soluble FGF-1 concentration.

**Table I.** Endothelial cell (EC) and smooth muscle cell (SMC) proliferation on fibrin glue (FG) plated in a delayed fashion. SMCs or ECs were plated on FG containing fibroblast growth factor-1 (FGF-1) and heparin 24 hours after the FG was made to exclude the effect on cell growth of the initial release of FGF-1 and heparin into the media. The data are presented as the percent of the negative control (FG with no heparin or FGF-1). N = 12 for each observation.

| | *EC proliferation (% control)* | | | *SMC proliferation (% control)* | | |
|---|---|---|---|---|---|---|
| *U/mL heparin* | *5* | *50* | *500* | *5* | *50* | *500* |
| No cytokine | 115 ± 3 | 86 ± 6 | 26 ± 30 | 118 ± 12 | 99 ± 21 | 78 ± 20 |
| 10 ng/mL FGF-1 | 144 ± 7*† | 107 ± 8* | 15 ± 12 | 156 ± 15† | 193 ± 28* | 161 ± 18* |
| 100 ng/mL FGF-1 | 220 ± 14*† | 116 ± 7* | 52 ± 91 | 325 ± 59*† | 392 ± 30* | 367 ± 37* |
| 1000 ng/mL FGF-1 | 228 ± 12* | 124 ± 7* | 10 ± 10 | 408 ± 38* | 393 ± 27* | 379 ± 45* |

*$P < .01$ versus same heparin dose with no cytokine.
†$P < .0001$ for 10 ng/mL FGF-1 versus 100 ng/mL FGF-1 with 5 U/mL heparin for both ECs and SMCs.

## DISCUSSION

FG has many applications,[9] including hemostasis in surgery[10,11] and thermal burns[12] and as a carrier for antibiotics[13] and chemotherapeutic agents,[14] in addition to its uses as a surface coating for vascular grafts[3,4,15] and angioplasty sites.[6] FGF-1 was chosen for its strong chemoattractant and mitogenic activity for ECs and its relatively weak SMC mitogenic activity. In the absence of heparin, FGF-1 induces minimal SMC proliferation. With the addition of small amounts of heparin, SMC proliferation dramatically increases. The addition of heparin, however, is necessary in FG to protect FGF-1 from thrombin-induced cleavage.[8] Previous reports[5,7] have used multiple concentrations of cytokines and heparin in FG in in vitro tissue culture models to optimize FG systems for use in animal models. Both of these papers found that high heparin concentrations (500 U/mL) in the FG inhibited SMC proliferation, but did not affect EC proliferation. These studies used immediate plating of cells and thus were affected by the soluble factors that are released from the FG. We have found that 70% ± 1% of FGF-1 and 59% ± 2% of heparin is released from the FG within the first 24 hours. To assess the effect of this release on cell growth, we placed media in the wells for 24 hours, and then removed the media before plating the cells (delayed plating). There was five times more $^{125}$I-FGF-1 and four times more $^{3}$H-heparin in the media of the immediate, as compared with the delayed, plating conditions, and the presence or absence of cells had minimal effects on the release of these substances from the FG. The data for the release of $^{3}$H-heparin from FG at the 500 U/mL concentration showed a higher percentage (80% ± 1%) than the 5 U/mL concentration (59% ± 2%) at 24 hours. There were methodological difficulties with measuring the release of the 500 U/mL concentration, because the FG was too hot to count accurately.

Quantifying the release of substances from FG is important for designing in vitro assays that may better predict the effect of the FG composition in vivo. One must be careful, however, not to extrapolate the in vitro release data to in vivo situations and different surfaces. In vivo rabbit studies with radiolabeled FGF-1 in FG impregnated onto ePTFE aortic interposition grafts revealed 45.1% ± 5.7%, 19.5% ± 1.6%, and 5.4% ± 0.2% retention after 5 minutes, 60 minutes, and 7 days, respectively.[16] By using a carotid balloon-injury model in dogs,[6] we studied the percentage retention of $^{125}$I-FGF-1 in FG on the injured arterial surface. After 10 minutes of arterial circulation, there was 41% retention of $^{125}$I-FGF-1 and 37.4% ± 12.2% after 60 minutes (no significant difference between the two time points).

By using the 24-hour in vitro release data, we then altered our proliferation assay to minimize the effect of the soluble substances on cellular proliferation by placing growth media on the FG for 24 hours before plating the cells. When plated in a delayed fashion, heparin at concentrations as high as 500 U/mL in the FG did not inhibit SMC proliferation. We then performed our assay with immediate plating of SMCs to see if the heparin inhibition of SMC proliferation reported by Kang et al[5] and Weatherford et al[7] could be reproduced. Heparin at a concentration of 500 U/mL in the presence of FGF-1 at 10, 100, or 1000 ng/mL again inhibited SMC proliferation, as compared with 5 U/mL heparin ($P < .001$ for all three FGF-1 concentrations). Comparing immediate versus delayed SMC plating, at equivalent FGF-1 and heparin doses,

**Table II.** Smooth muscle cell proliferation on fibrin glue plated in an immediate fashion. The data are presented as the percent of the negative control (fibrin glue with no heparin or fibroblast growth factor-1). N = 12 for each observation.

| | *Immediate plating: SMC proliferation (% control)* | | |
|---|---|---|---|
| *U/mL heparin* | *5* | *50* | *500* |
| No cytokine | 109 ± 10† | 98 ± 14‡ | 35 ± 23†‡ |
| 10 ng/mL FGF-1 | 498 ± 70*† | 421 ± 55* | 352 ± 24*† |
| 100 ng/mL FGF-1 | 598 ± 120*† | 585 ± 114* | 460 ± 22*† |
| 1000 ng/mL FGF-1 | 571 ± 106*† | 573 ± 102* | 450 ± 33*† |

*$P < .01$ versus same heparin dose with no cytokine.
†$P < .001$ for 5 U/mL heparin versus 500 U/mL heparin with the same fibroblast growth factor-1 concentration.
‡$P < .04$ for 50 U/mL heparin versus 500 U/mL heparin.
*SMC,* smooth muscle cell; *FGF-1,* fibroblast growth factor-1.

immediate plating induced greater proliferation than delayed plating ($P < .001$ for all FGF-1 and heparin combinations tested). As seen with the radio-labeled release experiments, immediate versus delayed plating resulted in five times more $^{125}$I-FGF-1 and four times more $^{3}$H-heparin in the media of the immediate plating. SMCs are very sensitive to soluble heparin and FGF-1 in the media. FGF-1 concentrations as low as 1 ng/mL with 5 U/mL heparin in the media can induce significant proliferation. Heparin at a concentration of 50 U/mL significantly decreases SMC proliferation in the presence of FGF-1, as compared with 5 U/mL heparin when both factors are soluble in the media. Heparin at a concentration of 500 U/mL in the media reduces SMC proliferation to baseline levels, even in the presence of 10 ng/mL FGF-1.[8] By using the radio-labeled data for immediate versus delayed plating to estimate the concentration of FGF-1 and heparin in the media caused by diffusive release, at the 100 ng/mL FGF-1 and 500 U/mL heparin doses in FG, the concentration of FGF-1 in the media would be 15 and 70 ng/mL in the delayed and immediate plating media, respectively. Heparin concentrations would be 70 and 295 U/mL in the delayed and immediate plating media, respectively. These concentration differences can explain the discrepancy between the heparin effect on SMC proliferation when immediate and delayed plating are used. We cannot entirely exclude, however, the effect of soluble FGF-1 in the media, even with the delayed method of plating. In vivo release data of substances from FG are different than the in vitro release data presented in the current work. In vivo, substances released from FG will diffuse into the blood vessel lumen and will be locally removed by blood flow. Diffusive loss will also occur in the vessel or graft wall FG impregnation setting.

EC proliferation with delayed plating revealed rather surprising results in comparison with the two earlier studies. In the current study, 50 U/mL heparin in the FG in the presence of FGF-1 inhibited EC proliferation, as compared with 5 U/mL heparin. As the heparin increased to 500 U/mL, the ECs died. In contrast, Weatherford et al[7] demonstrated that the addition of 50 U/mL heparin in FG enhanced EC proliferation (immediate plating) in the presence of VEGF, as compared with FG containing VEGF alone. Data for 500 U/mL heparin in FG were not shown in this paper for ECs. Kang et al[5] found that heparin at concentrations as high as 500 U/mL in the FG did not affect EC proliferation (immediate plating). The soluble heparin concentration in the Kang et al[5] study would be higher than in the current study because of the differences induced by immediate versus delayed cell plating. Perhaps these discrepancies can be explained by the cell types used in these three studies, which may reflect differences in response by ECs of different species or of different vascular beds. Weatherford et al[7] used human aortic ECs, and Kang et al[5] used HUVECs, whereas we used canine jugular vein ECs. We chose canine jugular vein ECs because we have previously seeded this cell type contained within FG onto balloon-injured canine carotid arteries.[6] Also, we wanted to use canine ECs because our in vivo model is in the dog. We can merely speculate on the differences reported from these three studies, because the data presented in this paper provide no clear answers to these questions. Perhaps the discrepancy in the three papers is caused by differences in the types of heparin used or differences in human

versus canine ECs. Alternatively, differences in venous versus arterial ECs may also play a role. HUVECs contain fetal components, and thus, this cell line does not always respond the same as adult ECs. We are currently culturing canine arterial ECs to see if the arterial ECs also display the same heparin sensitivity exhibited by the venous canine ECs. Thus, the results of this study vary significantly from the two earlier studies because of immediate versus delayed plating, differences in the heparin used, and differences in the cells used.

It is difficult to differentiate growth inhibition from cell death. We did not assay lactate dehydrogenase or other markers of cell death. However, neither SMC or EC death has been reported from FGF-1 or heparin in the concentrations used. Morphologically, the cells appeared intact, and minimal cellular debris or desquamation was observed under phase contrast microscopy in any experiment, with the exception of the ECs exposed to 500 U/mL heparin, in which cell loss and cellular debris were visible.

This study highlights the importance of the release of substances from FG and the importance of species differences when designing in vitro assays. For in vivo use in the dog model, this report supports the use of low heparin concentrations (5 U/mL) in the FG. The concentration of FGF-1 should be high enough to still be effective after the initial, large release of FGF-1 exhibited by both in vitro and in vivo[6,16] studies. Further experiments studying the release of radioactive FGF-1 from FG in the in vivo dog model are needed to address the issue of FGF-1 concentration. This, however, still does not address the issue of capsular thickening seen in the report by Gray et al[4] and the concerns about intimal hyperplasia induced by the FGF-1 proliferative effect on SMCs. By using site-directed mutagenesis, we are currently exploring the effect of different FGF-1 mutants on EC and SMC proliferation. We hope to discover a mutant that will preferentially stimulate EC proliferation while inhibiting, or at least not enhancing, SMC proliferation.

**REFERENCES**

1. Callow AD. Current status of vascular grafts. Surg Clin North Am 1982;62:501-13.
2. Clagget GP. Hemostasis and thrombosis: basic principles and clinical practice. In: Colman RW, Hirsch J, Marder VJ, Salzman EW, editors. Artifical devices in clinical practice. Philadelphia: JB Lippencott; 1987. p. 1348-65.
3. Greisler HP, Cziperle DJ, Kim DU, Garfield JD, Petsikas D, Murchan PM, et al. Enhanced endothelialization of expanded polytetrafluoroethylene grafts by fibroblast growth factor type 1 pretreatment. Surgery 1992;112:244-55.
4. Gray JL, Kang SS, Zenni GC, Kim DU, Kim PI, Burgess WH, et al. FGF-1 affixation stimulates ePTFE endothelialization without intimal hyperplasia. J Surg Res 1994;57:596-612.
5. Kang SS, Gosselin C, Ren D, Greisler HP. Selective stimulation of endothelial cell proliferation with inhibition of smooth muscle cell proliferation by fibroblast growth factor-1 plus heparin delivered from fibrin glue suspensions. Surgery 1995;118:280-7.
6. Zarge JI, Huang P, Husak V, Kim DU, Haudenschild CC, Nord RM, et al. Fibrin glue containing fibroblast growth factor type 1 and heparin with autologous endothelial cells reduces intimal hyperplasia in a canine carotid artery balloon injury model. J Vasc Surg 1997;25:840-9.
7. Weatherford DA, Sackman JE, Reddick TT, Freeman MB, Stevens SL, Goldman MH. Vascular endothelial growth factor and heparin in a biologic glue promotes human aortic endothelial cell proliferation with aortic smooth muscle cell inhibition. Surgery 1996;120:433-9.
8. Shireman PK, Hampton B, Burgess WH, Greisler HP. The FGF-1 mutant S130K induces heparin-independent smooth muscle cell proliferation and is resistant to thrombin degradation in fibrin glue. Surg Forum 1998;XLIX:348-50.
9. Shireman PK, Greisler HP. Fibrin sealant in vascular surgery: a review. J Long-Term Effect Med Implants 1998;8:117-32.
10. Milne AA, Murphy WG, Reading SJ, Ruckley CV. Fibrin sealant reduces suture line bleeding during carotid endarterectomy: a randomized trial. Eur J Vasc Endovasc Surg 1995;10:91-4.
11. Jakob H, Campbell CD, Qiu Z-K, Pick R, Replogle RE. Use of fibrin sealant for reinforcing arterial anastomoses. J Vasc Surg 1984;1:171-80.
12. McGill V, Kowal-Vern A, Lee M, Greenhalgh D, Gomperts E, Bray G, et al. Use of fibrin sealant in thermal injury. J Burn Care Rehabil 1997;18:429-34.
13. Thompson DF, Davis TW. The addition of antibiotics to fibrin glue. South Med J 1997;90:681-4.
14. Kitazawa H, Sato H, Adachi I, Masuko Y, Horikoshi I. Microdialysis assessment of fibrin glue containing sodium alginate for local delivery of doxorubicin in tumor-bearing rats. Biol Pharm Bull 1997;20:278-81.
15. Muller-Glauser W, Zilla P, Lachat M, Bisang B, Rieser F, von Segesser L, et al. Immediate shear stress resistance of endothelial cell monolayers seeded in vitro on fibrin glue-coated ePTFE prostheses. Eur J Vasc Surg 1993;7:324-8.
16. Greisler HP, Kang SS, Lin P, Hirko MK. In vivo and in vitro release kinetics of FGF-1 when impregnated into ePTFE grafts using fibrin glue suspensions. Trans Soc Biomater 1995;18:104.

Submitted Sep 24, 1998; accepted Feb 19, 1999.

## DISCUSSION

**Dr Joseph Schneider** (Evanston, Ill). I am intrigued by the bioengineering question of how long it would take these cytokines to elute from a graft or denuded artery in vivo and how that compares with the amount of time the material needs to be around in the petri dish to have a beneficial effect. Could it be around long enough to have a local beneficial effect in vivo?

**Dr Paula Shireman.** The kinetics of cellular proliferation in vitro are very different than in vivo proliferation.

In vitro, the greatest amount of proliferation in this mold occurs 24 to 72 hours after plating, and then proliferation decreases because of contact inhibition. In vivo kinetics would theoretically be different, because you are dealing with a denuded artery after an angioplasty or a graft surface in which contact inhibition would probably not occur within the first 72 hours. So, there are kinetic differences, and certainly we've not looked at what length of time is important for the fibroblast growth factor-1 (FGF-1) to be present. Whether a pulse dose of FGF-1 within the first hour with subsequent removal would cause similar proliferation versus having the FGF-1 there constantly, which is essentially what is happening in our model, has not been investigated by our laboratory. With the fibrin glue, our thought is that it's a slow-release type of system, which keeps the cytokine there longer than if you merely put the cytokine onto a graft site or into the media. And so, that's one of the reasons we've tried to use fibrin glue as a cytokine-release system, in an attempt to try to re-endothelialize denuded arteries and prosthetic grafts.

**Dr William Turnipseed** (Madison, Wis). I have a couple of questions. First, are there any kinetics for subset release that would be different in an in vitro, as opposed to an in vivo, model?

Second, were you aware of the species differentiation of response to heparin between the canine and human models? If so, why didn't you do this with human cells?

**Dr Shireman.** Those are two very good questions. I will answer the question about heparin effect on different types of endothelial cells first.

We were surprised by this too, because previous reports in the literature have used enough heparin to augment FGF-1 or whatever cytokine they were studying, which requires relatively low doses of heparin. I do not think there have been many studies that have looked at heparin toxicity in relation to endothelial cells, and not many people have looked at high heparin concentrations. The reason we were initially interested in high heparin concentrations was as a way to inhibit smooth muscle cells and yet not decrease the endothelial cell proliferation. The original studies that we did were on human umbilical vein endothelial cells, which are sort of a renegade breed of endothelial cells. We used them because they are readily available and easy to grow, but I am not sure that the response of the human umbilical vein endothelial cells really reflects what will happen in human or other animal models.

Why does heparin inhibit canine jugular vein endothelial cell proliferation? I don't know. It could be that there is something added to the heparin during processing that is actually inhibitory to the endothelial cell. Endothelial cells make heparinoids. But maybe too much of a good thing is not always better, and there appear to be toxic levels. Whether this reflects a venous versus an arterial difference or a human versus a canine difference, I'm not sure, and of all the observations that have come from this paper, that might be the most interesting question to chase.

About the question regarding the difference between in vivo and in vitro release kinetics: We do have some data that has been accumulated in our laboratory measuring in vivo release of radioactive FGF-1. In a rabbit model with polytetrafluoroethylene grafts coated with $^{125}$I-FGF-1 in fibrin glue implanted into the aorta, exposed to blood flow, and removed at different time points, the percentage retention of FGF-1 on the graft was 45% ± 5.7%, 19.5% ± 1.6%, and 5.4% ± 0.2% at 5 minutes, 60 minutes, and 7 days, respectively. If you compare that with the in vitro data, after 24 hours, 70% of the FGF-1 has been released into the media. So, certainly the FGF-1 may have some binding kinetics to the arterial lining or to the polytetrafluoroethylene grafts that we are not seeing in the in vitro fibrin glue kinetic studies.