Cellular Response to Transforming Growth Factor-β1 and Basic Fibroblast Growth Factor Depends on Release Kinetics and Extracellular Matrix Interactions

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The extracellular matrix plays an important role in growth factor biology, serving as a potential platform for rapid growth factor mobilization or a sink for concentrated sequestration. We now demonstrate that when a growth factor binds reversibly to the matrix, its effects are augmented by this interaction, and when the factor is absorbed irreversibly to the extracellular matrix, it becomes sequestered. These findings call into question the notion that all growth factors are best presented to cells and tissues in a sustained and controlled fashion. In our studies, we examined basic fibroblast growth factor (bFGF) and transforming growth factor-β1 (TGF-β1) release kinetics from synthetically fabricated microsphere devices and naturally synthesized extracellular matrix. While the sustained release of bFGF was up to 3.0-fold more potent at increasing vascular endothelial and smooth muscle cell proliferation than bolus administration, the reverse was true for TGF-β1. A bolus of TGF-β1 inhibited vascular cells up to 3.8-fold more efficiently than the same amount of TGF-β1 if control-released. Both growth factors bound to the extracellular matrix, but only bFGF was released in a controlled fashion (2.8%/day). Contact with the extracellular matrix and subsequent release enhanced bFGF activity such that it was 86% more effective at increasing smooth muscle cell numbers than equal amounts of growth factor diluted from frozen stock. TGF-β1 remained tightly adherent. The small amount of TGF-β1 released from the extracellular matrix was 30% less effective than bolus administration at inhibiting vascular endothelial and smooth muscle cell growth. Sustained growth factor release may be the preferable mode of administration, but only when a similar mode of metabolism is utilized endogenously.

The extracellular matrix is an integral part of growth factor biology. Many growth factors bind to this substrate, and it has been postulated that the matrix serves as a sequestration site from which growth factor stores can be concentrated for enhanced local action or released for heightened overall effect (1–7). Basic fibroblast growth factor (bFGF) is a model compound in this regard. Endogenous bFGF has been found localized to heparan sulfate-rich basement membranes in vivo (8, 9). The binding of bFGF to heparan sulfate proteoglycans protects bFGF from proteolytic degradation and may provide a reservoir of active bFGF in the extracellular matrix that may be available over a prolonged time (7, 10–15). The controlled release of bFGF might be important for maintenance of intact tissues, while acute injury might induce a burst release of bFGF following extracellular matrix degradation and tissue destruction. Accordingly, it has been assumed that the maximum biologic effect of bFGF and related growth factors is observed only when control-released. Indeed, we (16–18) and others (19–21) have demonstrated profound biologic effects of bFGF when control-released to cells in culture or tissues in vivo.

These findings have been extended to matrix-binding growth factors, and it is now well documented that cells optimally respond to the controlled release of growth factors (18, 22, 23). The exceedingly low doses, rapid clearance, and denaturation of these compounds even further support the idea that controlled or sustained release is an absolute requirement. A wide range of techniques have been created to ensure controlled delivery. Yet, few have investigated the validity of the underlying assumption that the most effective delivery of all growth factors to target cells and tissues is in a slow and controlled fashion, rather than a single bolus administration. This paradigm may not be true. Cells may paradoxically respond least well to sustained growth factor release. Consequently, this study sought to understand how, when, and why cells of the vasculature respond to different modes of growth factor administration and to compare effects obtained with bFGF with those obtained with transforming growth factor-β1 (TGF-β1).

Reports of growth factor physiology often compare bFGF and TGF-β1, for together they possess the fullest range of biochemical and biophysical parameters at opposite ends of the spectrum of biologic effects (24, 25). They both have short in vivo half-lives of <30 min (26–28) and yet have profound and sustained mediation of angiogenesis (29–32), gene expression (33–35), and extracellular matrix accumulation (36–39). Since cellular events such as receptor kinetics or ligand trafficking alone cannot explain the prolonged biologic effects and because both growth factors are matrix-binding, this interaction may be responsible for their sustained effects. We found, however, that while the sustained release of bFGF was up to 3.0-fold more potent at increasing vascular endothelial and smooth muscle cell proliferation than bolus administration, the reverse was true for TGF-β1. A bolus of TGF-β1 inhibited vascular cells up to 3.8-fold less effectively than equal amounts of TGF-β1 released from the extracellular matrix.
to 3.8-fold more efficiently than the same amount of TGF-β1 if control-released. Both growth factors bound to the extracellular matrix, but only bFGF was released in a controlled fashion. Contact with the extracellular matrix and subsequent release enhanced bFGF activity. bFGF released from the extracellular matrix was more effective at increasing vascular cell growth than equal amounts of growth factor diluted from frozen stock. TGF-β1 remained tightly adherent, and the small amount of the growth factor released from the extracellular matrix was less effective than bolus administration at inhibiting vascular cell growth. Sustained growth factor release may be the preferable mode of administration, but only when a similar mode of slow release metabolism is utilized endogenously. A more precise understanding of the relationship between the matrix and growth factors will not only aid in the design of formulations for therapeutics, but will also lead to an unveiling of a more definite pathophysiology of the diseases that growth factors govern.

EXPERIMENTAL PROCEDURES

**Cell Isolation**—Endothelial and smooth muscle cells were isolated from freshly excised aortas of 3–4-week-old calves (Area and Sons, Hopkinton, MA). Endothelial cells were harvested by incubating the luminal side of the aorta with a 1 mg/ml collagenase digestion (Sigma) for 90 min. The collagenase was discarded, and the cells were removed by gently washing the luminal side with a 0.5-mI aliquot of high antibiotic medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin (200 units/ml), streptomycin (200 μg/ml), 2% Fungizone, glutamine (100 mM), and 10% calf serum (all from Life Technologies, Inc.). The freshly isolated endothelial cells were placed in a 60 x 15-mm sterile Petri dish containing a sterile glass coverslip with 2.0 ml of high antibiotic medium and allowed to grow at 37°C in a humidified 5% CO2, 95% air incubator.

To isolate smooth muscle cells, the collagenase-digested luminal side of the aorta was cut open longitudinally and exposed. The luminal side of the aorta was cut open longitudinally and exposed. The freshly isolated endothelial cells were placed in a 60 x 15-mm sterile Petri dish containing a sterile glass coverslip with 2.0 ml of high antibiotic medium and allowed to grow at 37°C in a humidified 5% CO2, 95% air incubator.

**Cell Maintenance**—Both cell lines were subcultured with 0.25% trypsin, 1 mM EDTA in Hanks’ balanced salt solution (Life Technologies, Inc.) when the cells were subconfluent. The cells were maintained in 75-cm² culture flasks (Costar Corp.) in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), glutamine (100 mM), and 10% calf serum. Both cell lines were cultured up to passage 5. Cell numbers were determined by counting trypsinized cells with a ZF1 Coulter Counter.

**Cell Proliferation Assays**—The differential effects of bolus or control-released amounts of bFGF or TGF-β1 on the proliferation of sparsely seeded (4 x 10⁵ cells/ml/well) endothelial and smooth muscle cells were examined in 2.5-cm cluster 12-well tissue culture-treated plates (Costar Corp.). Endothelial cells were plated in DMEM containing 10% calf serum and allowed to attach overnight. Smooth muscle cells were plated in DMEM containing 5% calf serum, allowed to attach overnight, and then growth-arrested by changing the medium to 0.05% calf serum for 3 days.

bFGF proliferation assays were performed by changing the culture medium of 24-h endothelial or growth-arrested smooth muscle cells to DMEM containing 1% calf serum. Alginate/heparin-Sepharose controlled release microspheres with or without bFGF were placed into the culture wells with sterile forceps, or equivalent amounts of concentrated bFGF were added directly to the culture medium as a single bolus. TGF-β1 assays were performed by replacing the culture medium of 24-h endothelial or growth-arrested smooth muscle cells with fresh DMEM containing 10% calf serum. Ethylene-vinyl acetate copolymer (EVA)-bovine serum albumin (BSA)-TGF-β1 controlled release microspheres with and without TGF-β1 were placed into the culture wells with sterile forceps, or equivalent amounts of concentrated TGF-β1 were added directly to the culture medium as a single bolus or bolus additions constituted day 1 of the proliferation assay. For both assays, cells were allowed to grow for the indicated times, and cell number was determined by trypsinization followed by number assessment with a ZF1 Coulter Counter.

**Alginate/Heparin-Sepharose Microsphere Preparation and Growth Factor Incorporation**—Alginate/heparin-Sepharose microspheres were prepared according to methods previously described (18, 22). Briefly, a 1.8% solution of low molecular weight sodium alginate (Sigma) was prepared in water and sterilized by filtration. Heparin-Sepharose beads (CL-6B, Pharmacia Biotech Inc.) were sterilized under ultraviolet light and allowed to swell in sterilized water. After three washes in water, the beads were resuspended in water (1:1, v/v). The sodium alginate solution and heparin-Sepharose beads were mixed with a final concentration of 33.3 mg/ml heparin-Sepharose and 1.2% (w/v) sodium alginate. This mixed suspension was dropped through a sterile Pasteur pipette into a 500-ml gently stirring solution of 1.5% CaCl2. Upon entering the CaCl2 solution, the alginate/heparin-Sepharose suspension immediately formed hard spherical pellets. These microspheres were mixed gently for 5 min to cure and were then allowed to incubate in PBS at room temperature for 10 min to slow release metabolism. The microspheres were washed three times in sterile water and stored at 4°C in 150 mM NaCl and 1 mM CaCl2 for up to 3 months.

bFGF incorporation into the alginate/heparin-Sepharose microsphere was performed by first placing the microspheres in sterile microcentrifuge tubes coated with 150 mM NaCl, 1 mM CaCl2, and 0.5% gelatin (18, 22). The beads were mixed with a 1-μl aliquot of bFGF (100 μg/ml; R&D Systems, Minneapolis, MN) or Bolton-Hunter labeled human recombinant bFGF (70 μCi/μg; DuPont NEN) was added along with 14 μl of 150 mM NaCl, 1 mM CaCl2, and 0.15% gelatin. The beads were gently agitated overnight at 4°C. bFGF release studies were performed as described by Nugent et al. (18) using three alginate/heparin-Sepharose microspheres.

**EVA Microspheres Preparation and Growth Factor Incorporation**—EVA microspheres were prepared according to the methods of Setton et al. (40). Briefly, EVA (Elvax 40, DuPont) was washed thoroughly in water and ethanol and then dissolved in methylene chloride to yield a 10% (w/v) solution. BSA was weighed out to provide a 30% loading concentration (BSA/EVA, v/w) in glass scintillation vials and was dissolved in 2 ml of Milli-Q water. TGF-β1 (R&D Systems) or Bolton-Hunter labeled human recombinant TGF-β1 (120–180 μCi/μg; DuPont NEN) was added to this suspension to yield equal ng of TGF-β1/microsphere. The solution was lyophilized to a dry powder and crushed fine, and dissolved 10% EVA solution was then added to the powder. This mixture was then dropped using a Pasteur pipette into a glass beaker filled with 50 ml of ethanol (−40°C) that was placed by immersion in dry ice-ethanol solution in which there was an excess of EVA solution. Hard spherical pellets formed immediately as the mixture entered the cold ethanol solution. After 5–10 min, the ethanol solution containing the EVA microspheres was allowed to warm to room temperature. The ethanol was changed, and the microspheres were allowed to cure overnight at ambient temperature. The next day, the ethanol was decanted, and any residual ethanol was removed by lyophilization. After the removal of the ethanol, the microspheres were kept under sterile conditions at room temperature. TGF-β1 release kinetics were followed for 5–10 EVA-BSA-3H-bFGF microspheres placed in 1 ml of PBS at room temperature with gentle shaking. At designated times, the PBS containing the released 3H-bFGF was removed and replaced with a fresh 1-ml PBS aliquot for 10 days. The amount of radioactivity was assessed using a Pharmacia Biotech scintillation counter.

**Extracellular Matrix Incorporation and Release of Growth Factors**—Endothelial or smooth muscle cells were plated at 1 x 10⁴ cells/ml/well in 2.5-cm cluster 12-well tissue culture-treated plates using DMEM supplemented with glutamine, antibiotics, and 10% calf serum. Cells reached confluency in ~7 days. The confluent monolayers were washed one time in PBS (Ca²⁺- and Mg²⁺-free) and incubated with 1 ml/well of 1 mM Mg²⁺-free extraction buffer (0.5% Triton X-100, 20 mM NH₄OH in Ca²⁺- and Mg²⁺-free PBS) for 5 min at room temperature to solubilize the cells. The extra buffer was removed, and the wells were washed five times with PBS (Ca²⁺- and Mg²⁺-free) to remove the solubilized cells, leaving extracellular matrix coating the bottom of the wells (41). The extracellular matrix-coated wells were reconstituted with 1 ml of Ca²⁺- and Mg²⁺-free PBS and retained at 37°C until continuing with subsequent procedures.

bFGF, TGF-β1, and/or 125I-TGF-β1 was incorporated into the extracellular matrix by first incubating the matrix with 1 ml of blocking buffer (DMEM, 50 mM Hepes, pH 7.4, containing 0.05% gelatin) for 10 min at room temperature. The blocking buffer was discarded, and

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another 1-ml aliquot of the blocking buffer was placed on the matrix. Growth factor at 1–10 ng/ml concentrations was added and then incubated at 4 °C for 2 h. The buffer solution was removed, and the growth factor-incorporated extracellular matrix was washed three times with fresh blocking buffer at 4 °C. To determine the amount of incorporated growth factor, the matrix from the ³²P-bFGF or ¹²⁵I-TGF-β₁ incorporation was removed by placing 1 ml of 1 N NaOH in the well and incubating the well for 5 min at room temperature. The NaOH solution containing the suspended extracellular matrix with incorporated ¹²⁵I-growth factor was counted using a Pharmacia Biotech 1272 CliniGamma γ-counter.

In other identical plates, the release of ¹²⁵I-bFGF or ¹²⁵I-TGF-β₁ from the extracellular matrix was monitored. Wells were covered with 1 ml of release buffer (DMEM, 50 mM Hepes, pH 7.4, containing 0.15% gelatin) at 37 °C. At designated times, the buffer now containing released growth factors was removed and replaced with fresh 1-ml aliquots of release buffer. The amount of radioactivity in each aliquot was assessed by γ-counting.

Nonradioabeled bFGF or TGF-β₁ (1–10 ng/ml) in blocking buffer was exposed to the extracellular matrix for 2 h at 4 °C. The buffer solution was removed, and the extracellular matrix was washed three times with fresh blocking buffer at 4 °C. Fresh release buffer was placed on the wells, retrieved after 1 h at 37 °C, replaced with a fresh 1-ml aliquot, and then removed after the second hour. The second-hour aliquot represented growth factor bound and subsequently released from the extracellular matrix. This aliquot was transferred onto PBS-washed endothelial or smooth muscle cells plated at 4 × 10⁵ cells/well. Cells were allowed to grow for the indicated times, and cell number was determined by trypsinization followed by number assessment with a ZF1 Coulter Counter. These results were compared with cells exposed to equivalent amounts of growth factor diluted from frozen stocks.

Statistics—All data are expressed as the mean ± S.E. of at least triplicate wells or vials. Statistical comparisons were performed using Student’s t test or analysis of variance where appropriate. Data were statistically significantly different when p values were <0.05.

RESULTS

Release Kinetics—Controlled release devices were constructed to release TGF-β₁ and bFGF with similar kinetics. Two different devices were used because TGF-β₁ is not significantly heparin-binding (42), and bFGF is denatured by the methylene chloride (18, 22) required to solvent-cast EVAc release devices (40). Virtually all (>94.1%) of the bFGF was denatured by the organic solvents used to fabricate EVAc matrix devices (18, 22), while TGF-β₁ retained its biologic activity when exposed to methylene chloride fumes. When compared with controls, there was no statistically significant difference in the inhibition of endothelial cells (78.1%, p < 0.0001) by TGF-β₁ or methylene chloride-treated TGF-β₁ (85.0%, p < 0.0001).

bFGF was released with first order kinetics from heparin-Sepharose beads embedded within calcium alginate microspheres. Repeated binding of bFGF to immobilized heparin encapsulated in the microspheres provided for sustained kinetics (Fig. 1). TGF-β₁ did not bind significantly to the heparin, and its release from these beads was too rapid to be of interest. When TGF-β₁ was solvent-cast within EVAc microspheres, first order release kinetics were observed, which were consistent with previous studies (40). Approximately 12% of the compound released within the first 8 days (Fig. 1). Alginate/heparin-Sepharose microspheres without bFGF incorporation and EVAc-BSA-TGF-β₁ microspheres without TGF-β₁ incorporation had no effect on cell growth (p = not significant) (data not shown).

bFGF—Alginate/heparin-Sepharose microspheres with and without bFGF were placed in wells containing sparsely plated (4 × 10⁵ cells/well) endothelial and smooth muscle cells. The controlled release of bFGF significantly increased endothelial cell proliferation beyond that observed with bolus infusion (Fig. 2A). While the bolus increased cell number 1.8-fold from control values of 3.4 ± 0.12 × 10⁵ cells/ml to 6.0 ± 0.23 × 10⁵ cells/ml (p < 0.0001), microspherical controlled release increased cell number 3.5-fold to 1.2 ± 0.04 × 10⁶ cells/ml (p < 0.0001). Smooth muscle cells demonstrated an even greater proliferative response to the sustained release of bFGF in comparison with an equivalent bolus infusion (Fig. 2B). The controlled release of bFGF increased cell number 6.5-fold over control...
values (3.0 ± 0.25 × 10^5 cells/ml versus 4.6 ± 0.14 × 10^5 cells/ml; p < 0.0001), while bolus stimulation produced only a 2.2-fold increase (1.0 ± 0.01 × 10^6 cells/ml; p < 0.0070).

As we (18, 22) and others (43) have previously demonstrated, polymeric device encapsulation can reduce growth factor biologic activity if an inappropriate choice of materials and formulations is used. When bFGF was embedded within and released from the polymeric devices used, the growth factor remained as biologically effective as equivalent compounds diluted from identical stock concentrations throughout the time course of the release experiments. The 0.3 ng of bFGF released from the alginate/heparin-Sepharose microspheres between 0.5 and 1 h produced a 2.9-fold increase in cell number over control values (2.3 ± 0.10 × 10^5 cells/ml versus 7.9 ± 0.17 × 10^5 cells/ml; p < 0.0001), while the same amount of bFGF released between days 5 and 6 demonstrated a 3.0-fold increase in cell number over control cells (2.1 ± 0.18 × 10^6 cells/ml versus 7.1 ± 0.34 × 10^6 cells/ml; p < 0.0001).

**TGF-β1**—TGF-β1 is a potent inhibitor of endothelial cell proliferation (Fig. 3A). A bolus dose as little as 0.4 ng/ml well demonstrated a 10.0-fold inhibition of cell proliferation. Control endothelial cells grew 47.5-fold (1.9 ± 0.15 × 10^6 cells/ml) in 8 days from plating densities of 4 × 10^3 cells/ml well (p < 0.0001). A 0.4-ng bolus of TGF-β1 restrained cell growth to only a 4.75-fold increase (1.9 ± 0.03 × 10^6 cells/ml; p < 0.0001). Control-released TGF-β1 was a 4-fold less stimulus to endothelial cells than bolus administration. One EVAc-BSA-TGF-β1 microsphere releasing 0.4 ng of TGF-β1 demonstrated only a 2.6-fold inhibition of cell proliferation (p < 0.0001) and allowed for an 18.0-fold increase (7.2 ± 0.25 × 10^5 cells/ml) in cell number over the original plating density (p < 0.0001). Smooth muscle cells were not as strongly inhibited by TGF-β1 as were endothelial cells, but exhibited an even greater differential effect with mode of delivery (Fig. 3B). A 0.4 ng/ml well bolus demonstrated a 1.8-fold inhibition of smooth muscle cell proliferation. While control smooth muscle cells with no TGF-β1 increased 115.0-fold (4.6 ± 0.2 × 10^5 cells/ml) above original plating densities of 4 × 10^3 cells/ml (p < 0.0001), the bolus allowed for cell numbers to increase only 65.0-fold (2.6 ± 0.02 × 10^6 cells/ml; p < 0.0001). The controlled release of TGF-β1 from an EVAc-BSA-TGF-β1 microsphere did not demonstrate a statistically significant effect on cell growth. Cell number was not changed from control values (4.5 ± 0.15 × 10^5 cells/ml).

Released TGF-β1 also retained its biologic activity throughout the experiment. The 0.03 ng of TGF-β1 released after the initiation of the experiment at 1 h produced an 11.2-fold decrease in cell number over control values (2.5 ± 0.29 × 10^4 cells/ml versus 2.8 ± 0.09 × 10^5 cells/ml; p < 0.0001). The same amount of TGF-β1 released at the end of the experimental kinetics profile demonstrated a statistically indistinguishable 10.8-fold decrease in cell number when compared with control values (4.0 ± 0.22 × 10^5 cells/ml versus 4.3 ± 0.47 × 10^5 cells/ml; p < 0.0001).

**Sequential TGF-β1 Bolus Administration**—To determine if the inhibitory effects of a large bolus administration of TGF-β1 could be achieved by the additive effects of smaller bolus additions, smooth muscle cells were exposed to either a 2.5-ng bolus of TGF-β1 or 0.5-ng TGF-β1 bolus additions for 5 consecutive days. Cell number was assessed the following day (Fig. 4). On day 6, smooth muscle cells exposed to the single large bolus already showed a 1.6-fold inhibition of cell proliferation compared with control cells (4.8 ± 0.1 × 10^5 cells/ml versus 7.6 ± 0.3 × 10^5 cells/ml; p < 0.0001). Cells exposed to consecutive small bolus additions were not inhibited at all and actually showed a slight 1.3-fold increase in cell number over control values (9.5 ± 0.2 × 10^5 cells/ml; p < 0.0014). An approximately equal, single bolus amount of TGF-β1 (0.4 ng) inhibited smooth muscle cell proliferation 1.8-fold (Fig. 3B), but if this approximate bolus amount (0.5 ng) was administered in a controlled release fashion, the inhibitory effect of TGF-β1 was not observed (Fig. 4).

**bFGF and TGF-β1 Release from the Extracellular Matrix**—125I-bFGF was incorporated into the extracellular matrix and then released over a 10-day time period (Fig. 5). Smooth muscle cell extracellular matrix was used as a representative matrix, which incorporated 10% of 125I-bFGF. After an initial burst, 125I-bFGF was gradually released in a sustained and predictable fashion at an average rate of 2.8%/day, with a cumulative release of 69% at day 10. 125I-TGF-β1 was incorporated with
far higher efficiency (38%), but remained bound to the matrix. Only 1.1% of the growth factor was released per day (Fig. 5). Similar results for $^{125}$I-TGF-$\beta_1$ were observed using endothelial cell extracellular matrix. The matrix incorporation of radiolabeled growth factors is representative of nonradiolabeled growth factor matrix incorporation.

Growth factor interaction with the extracellular matrix determined biologic activity. bFGF released from an extracellular matrix from the first to second hour of release after incorporation demonstrated a 43% increase in smooth muscle cell proliferation compared with control values ($p < 0.0001$) (Fig. 6). An equivalent bolus amount of growth factor diluted from frozen stock produced a 6% increase in cell proliferation compared with control values ($p < 0.0001$). The amount of bFGF released from the extracellular matrix was below the minimal dosage required to elicit an endothelial cell response.

The biologic activity of TGF-$\beta_1$ was retained after interaction with the extracellular matrix, but the ability of matrix-released TGF-$\beta_1$ to elicit its biologic effect decreased in contrast to matrix-released bFGF. Endothelial and smooth muscle cell proliferation was inhibited 62 and 67%, respectively, by bolus amounts of TGF-$\beta_1$, but was inhibited only 47 and 46%, respectively, by extracellular matrix-released TGF-$\beta_1$ (Fig. 7). Bolus and controlled release groups were statistically different from each other for both cell types ($*,$ endothelial cells, $p < 0.003$; $**,$ smooth muscle cells, $p < 0.0001$).

To preclude the possibility that the extracellular matrix itself would contribute to stimulatory or inhibitory effects by releasing its own endogenous growth factors, endothelial and smooth muscle cells were exposed to release buffer from smooth muscle cell extracellular matrix without the addition of exogenous bFGF or TGF-$\beta_1$. Cell growth was not statistically different from cells that were grown in medium alone (data not shown).

**DISCUSSION**

The binding and release of growth factors from the extracellular matrix have been proposed as a means of protecting, sequestering, and optimizing the biologic effect of these compounds (7, 10–15). It is now accepted that cell regulation requires controlled and sustained release; consequently, great efforts have been directed at ensuring prolonged kinetics of administration (21, 22, 44). In fact, growth factors have become the favorite compound with which to evaluate the efficacy of polymeric controlled drug delivery systems (18, 22, 23). To date, however, few have verified and virtually none have questioned the underlying assumption that sustained presentation

**FIG. 5.** Controlled release of bFGF and TGF-$\beta_1$ from the extracellular matrix. Shown is the percent cumulative release of $^{125}$I-bFGF ($\bullet$) or $^{125}$I-TGF-$\beta_1$ ($\square$) during a 10-day time period. Physical release of the growth factor was determined by measuring the presence of $^{125}$I-bFGF or $^{125}$I-TGF-$\beta_1$ in the release buffer from smooth muscle cell extracellular matrix incorporated with $^{125}$I-bFGF or $^{125}$I-TGF-$\beta_1$. Each data point represents the mean ± S.E. of three identical wells.

**FIG. 6.** Cellular response to bFGF released from the extracellular matrix. bFGF was incorporated into smooth muscle cell extracellular matrix and released into buffer that was collected every hour and replaced with fresh buffer. The aliquots collected during the second hour of release (○) were placed on smooth muscle cells plated at $4 \times 10^3$ cells/ml/well along with equivalent bolus amounts from frozen stock (□). Cells were counted on day 7. Each data bar represents the mean ± S.E. of three identical wells. Differences between extracellular matrix release and bolus groups are significant ($p < 0.0001$).

**FIG. 7.** Cellular response to TGF-$\beta_1$ released from the extracellular matrix. TGF-$\beta_1$ was incorporated into smooth muscle cell extracellular matrix and released into buffer that was collected every hour and replaced with fresh buffer. The aliquots collected during the second hour of release (○, □) were placed on endothelial (○, □) and smooth muscle (□, □) cells plated at $4 \times 10^3$ cells/ml/well along with equivalent bolus amounts from frozen stock (□, □). Cells were counted on day 8. Each data bar represents the mean ± S.E. of three identical wells. Differences between extracellular matrix release and bolus groups for both cell types are significant ($*, p < 0.0014$; $**, p < 0.0001$).

is the optimum means by which to regulate cell growth. We now demonstrate that for some growth factors sustained delivery is a far less desirable mode of delivery than bolus administration. While the sustained release of bFGF was up to 3.0-fold more potent at increasing vascular endothelial and smooth muscle cell proliferation than bolus administration, the reverse was true for TGF-$\beta_1$. A bolus of TGF-$\beta_1$ inhibited vascular cells up to 3.8-fold more efficiently than the same amount of TGF-$\beta_1$ if control-released. Both growth factors bound to the extracellular matrix, but only bFGF was released in a controlled fashion (2.8%/day). TGF-$\beta_1$ was absorbed within the matrix and remained tightly adherent. Contact with the extracellular matrix and subsequent release enhanced bFGF mitogenicity, but diminished TGF-$\beta_1$ activity. Thus, it appears that sustained growth factor release may be the preferable mode of administration only when a similar mode of metabolism is utilized endogenously. Reversible binding to the matrix may indeed signal an endogenous form of controlled release, but this phenomenon must be distinguished from irreversible absorption.

**Model of Events—**bFGF is exceedingly sensitive to denaturation (7, 12), lacks a signal sequence, and must interact with its receptor for up to 12 h to have its full biologic effect (45, 46). Some alternative mechanism must therefore exist for the presentation of this growth factor to the cell, and matrix binding and release have been suggested as fulfilling this role. bFGF binds avidly to heparan sulfate proteoglycans (5–9, 11, 21, 47). These complexes, which are richly abundant within the extra-
cellular matrix, are released by cells. The heparan sulfate proteoglycans protect bFGF from degradation (7, 10, 12), facilitate distribution (4) and kinetics (47), enrich biologic activity (1, 4, 14, 47), and enable its binding to specific tyrosine kinase receptors (15, 48–50). Thus, our observations that bFGF effects are enhanced after exposure to the extracellular matrix and when control-released probably reflect the protection and stabilizing effects of soluble proteoglycans and the depot potential of the matrix proteoglycans. The physicochemical properties and receptor kinetics of bFGF presumably led to the evolution of these natural protective and facilitory mechanisms and support the need for synthetic means of controlled release when consideration is given to their exogenous administration.

In contrast, the reverse is probably true for TGF-β1. Sustained release is a far less optimal means of administering this growth factor than bolus injection, and its matrix binding likely reflects a very different physiology compared with bFGF. Like bFGF, TGF-β1 has a short half-life and is prone to denaturation. The charge and hydrophobic properties that make it prone to degradation enable high affinity binding to extracellular matrix components such as type IV collagen, fibronectin, and the proteoglycans (51–55). Yet, TGF-β1 exists in a latent form that is less sensitive to destruction, and it reaches half-maximal receptor binding within 30–60 min and saturation within 4 h (53). Thus, at the outset, one can already imagine that sustained release is far less of an imperative. Matrix binding may therefore have arisen to address a different set of issues. Indeed, it is the latent form of the growth factor that interacts most with the extracellular matrix. Dimers of TGF-β1 capped by TGF-β1 latency-associated protein can bind to the specific latent TGF-β1-binding protein through disulfide linkage. Latent TGF-β-binding protein is rapidly secreted from cells, and the major fraction is covalently associated with the extracellular matrix. The 3-fold complex structure of latent TGF-β1, TGF-β1 latency-associated protein, and latent TGF-β1-binding protein can then bind to the matrix, where it has been postulated that it serves to target the growth factor to the extracellular matrix and create pools of latent TGF-β1 that act as negative feedback regulators. Indeed, we now show that unlike bFGF, extracellular matrix-bound TGF-β1 is not released to any discernible extent, even though binding to the TGF-β1 receptor is reversible (53). Proteolytic cleavage can release all of the three components of the TGF-β1 complex, yet like TGF-β1 interaction with heparin (42), interaction with the matrix does not augment biologic effect.

Thus, both bFGF and TGF-β1 bind to the extracellular matrix, but while the former is released in an augmented form in a prolonged manner, the latter is bound unless cleaved, and its association may even diminish its effects. Endogenous matrix binding may therefore protect, sequester, and then release bFGF, but also permanently bind TGF-β1 to target this growth factor to the matrix and act as a form of feedback regulation. Cells also recognize these two growth factors differently. Vascular endothelial and smooth muscle cells respond optimally to the continuous presentation of the growth factor that is naturally continuously released and to bolus administration of the growth factor that is irreversibly matrix-bound.

Controlled Release of Growth Factors—These data have profound ramifications for the huge industry that has been spawned for the controlled release of growth factors (23). Growth factors were the first compounds used to demonstrate the efficacy of polymer-based controlled release devices. Innovations in polymer chemistry and materials science technology that enabled the design and formulation of controlled release devices occurred at the same time that growth factors were being identified, characterized, purified, and cloned. Moreover, growth factors were ideal compounds to demonstrate the biologic effect of release devices because they are rapidly denatured, active in minute quantities, often difficult to isolate, and of great potential clinical and scientific benefit. Controlled delivery of epidermal growth factor (56, 57) has been reported to stimulate fibroblast proliferation (58), wound healing (59, 60), angiogenesis (61), and neovascularization (62). Recombinant platelet-derived growth factor has been released from collagen discs to promote tissue generation (63). Sustained release of nerve growth factor has been shown to stimulate neurite sprouting from PC12 cells (64), to prevent choline acetyltransferase loss (65, 66), and to prolong survival of adrenal medullary and other brain tissue (67, 68). The effects of control-released insulin on fibroblast proliferation have also been reported (58). TGF-β1 (69–71) and bone-derived growth factors that contain TGF-β1 (72, 73) have been utilized in controlled release systems in models of bone and wound healing (59, 74–77), angiogenesis (78), regulation of ocular growth (79), regulation of mammary gland (43) and epithelial cell (80) growth, and inducible expression from intracardiac grafts to the heart (81). The controlled release of bFGF has been shown to be effective in cell culture (18, 20, 82) as well as angiogenic in corneal tissue (61, 83), retinal regeneration (84), and peripheral nerve regeneration (85). Yet, few of these studies compared the effects of sustained release formulations and bolus delivery. Various polymeric devices have been used for the controlled release of a number of growth factors. A commonly used system for growth factor release is ethylene-vinyl acetate copolymer (40, 43, 61, 62, 64, 65, 67, 84–87), although the organic solvents used in the preparation of this device destroy the biologic activity of molecules such as bFGF (18, 22). TGF-β1 has been delivered for the purpose of bone repair via the biodegradable polymer poly-(DL-lactide-co-glycolide) and demineralized bone matrix (69, 71, 70, 73), although problems of immunocompatibility, osteoinductivity, and osteoconductivity exist. Other polymer materials have included poly(methyl methacrylate) (58), Pluronic F-127 poloxamer gel (74), polyethylene glycol (88), collagen (63, 77, 89), methylcellulose (75), chitosan (19, 82), cyclodextrin (20), lipids (90), Hydron (78, 83, 91), and other hydrogels, but these all have various limitations such as shorter or suboptimal release times and difficulty of handling. Microspheres consisting of the biocompatible and biodegradable polymer alginate have also been utilized for controlled release delivery of some growth factors. Alginate-polysylsine-alginate microspheres were used for the delivery of nerve growth factor (66), and sodium alginate microspheres for the delivery of endothelial cell growth factor (57) and bFGF (18, 22, 86). TGF-β1 release from sodium alginate microspheres has been demonstrated as a potential oral gastrointestinal drug delivery system, in which TGF-β1 is completely and rapidly released within 2 h after a low pH environment is changed to pH 7.4 (80). Our data now support the notion that in considering the burgeoning technology of controlled release, the different interactions these growth factors have with the extracellular matrix must be taken into account. Sustained release should be reserved for those growth factors that are naturally sustained-released.

Conclusion—These data illustrate that the relative functional potencies of growth factors are not dictated by molecular interactions alone, but by other parameters such as the absolute number of receptors, the ability of receptor down-regulation (92), growth factor availability (93, 94), and requirements for receptor activation. Growth factors may bind reversibly or absorb irreversibly to the extracellular matrix in response to very different needs and with very different consequences. This adds flexibility to the cell, enabling it to control the concentra-
tion of drug at its surface by regulating the kinetics of drug presentation and possibly surface receptor expression in concert with the more standard resolution achieved through local dose and binding affinity. Continued elucidation of these interactions will enhance our understanding of growth factor physiology (95, 96), e.g. to include the cellular processing of the released growth factors by living cells and tissue. Only by examination of the natural means by which endogenous mediators of growth are metabolized can we fully understand growth factor biology, derive insight into the pathophysiology of proliferative diseases, and provide rational means for their administration and appropriate rules for construction of devices for their controlled and sustained release if indicated.

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