Journal of Cellular Biochemistry 58:328–343 (1995)

Regulation of Basic Fibroblast Growth Factor (bFGF) Gene and Protein Expression Following Its Release From Sublethally Injured Endothelial Cells

Po-Tsan Ku and Patricia A. D'Amore

Program in Cell and Developmental Biology (P.-T.K., P.A.D.), and Department of Pathology (P.A.D.), Harvard Medical School; and Laboratory for Surgical Research (P.-T.K., P.A.D.), The Children's Hospital, Boston, Massachusetts 02115

Abstract Basic fibroblast growth factor (bFGF; FGF-2) lacks a signal sequence and thus is not secreted by classical pathways. It has been speculated that one mode of bFGF release may be injury, either sublethal or lethal; and, transient disruption of the plasma membrane has been shown to release bFGF [Muthukrihnan et al. (1991): J Cell Physiol 148:1–16]. This observation has led to the concept of bFGF as a "wound hormone," involved in tissue integrity and repair. Findings of elevated bFGF following injury in vivo support this concept. Using an in vitro model, we have examined the regulation of bFGF gene expression following its release by sublethal injury. Analysis of bFGF protein by ELISA revealed that scraping subconfluent bovine aortic EC (BAE) released up to 80% of their bFGF. Following scraping, there was a 4- to 10-fold increase in the steady state level of bFGF mRNA, which reached a maximum at 2–3 h. There was a parallel increase in protein so that by 6 h after the scrape-induced release, bFGF levels were restored to those measured prior to scraping. Since bFGF has been reported to induce its own expression, we hypothesized that the released bFGF might be responsible for the increase in bFGF mRNA. However, inclusion of neutralizing antibodies against bFGF had a negligible effect on the scrape-induced increase in bFGF mRNA levels. Because of the important role of transforming growth factor type-beta 1 (TGF-β1), the plasminogen/plasminogen activator system, and thrombin in wound healing, we investigated their potential contributions to the increase in bFGF expression. Addition of anti-TGF-β1 antibodies, plasminogen activator inhibitor-1 (PAI-1), or the thrombin inhibitory combination of heparin and anti-thrombin III (AT III) to the cells at the time of scraping blocked about 50% of the increase in bFGF mRNA; the effects of these agents were not additive. The suppression of bFGF mRNA was associated with a proportional reduction in bFGF protein. Inclusion of the antagonists for 2 h at the time of scraping led to reduced cell proliferation, suggesting that cell-associated bFGF may be required for recovery and growth. Finally, studies to characterize the molecular mechanisms underlying the increased bFGF mRNA following sublethal injury revealed an increase in the transcriptional activation of bFGF gene. These results indicate that in spite of the fact that bFGF is not a secreted protein, levels of bFGF in the cell are tightly regulated. Furthermore, these findings suggest a role for bFGF in recovery from cell injury. © 1995 Wiley-Liss, Inc.

Key words: growth factor, secretion, thrombin, TGF-β, plasmin, transcription

The fibroblast growth factors (FGFs), a family of polypeptides that were first purified from tumors as angiogenic agents and mitogens for endothelial cells (EC) [for review see Klagsbrun and D'Amore, 1991], are widely distributed and have a broad range of target cells of mesenchymal and neuroectodermal origin. Basic fibroblast growth factor (bFGF; FGF-2), a prototypic member of the FGF family, has been shown to be a potent regulator of vascular EC in vitro and in vivo; it is mitogenic and chemotactic for EC and increases their production of proteases [Moscatelli et al., 1986; Sato and Rifkin, 1988; Schweigerer et al., 1987].

In light of its pluripotency in many biological systems, it is perplexing that bFGF does not possess a signal peptide to direct secretion [Abraham et al., 1986]. It has been proposed that

Abbreviations used: BAE, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; EC, endothelial cells; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF-β, transforming growth factor type-beta.

Received August 10, 1994; accepted November 10, 1994.

Address reprint requests to Patricia A. D'Amore, Laboratory of Surgical Research, The Children's Hospital, 300 Longwood Avenue, Boston, MA 02115.

© 1995 Wiley-Liss, Inc.

bFGF is released from dead, damaged, or injured cells by leakage or lysis [D'Amore, 1990; Klagsbrun and Vlodavsky, 1988; Thomas, 1988]. Several lines of experimental evidence support this hypothesis. Treatment of bovine aortic EC (BAE) with endotoxin releases bFGF [Gajdusek and Carbon, 1989]. De novo synthesis and release of bFGF have been reported to occur as part of the cellular responses to irradiation [Witte et al., 1989]. Finally, creation of transient tears in the plasma membrane of EC, causing sublethal injury, leads to the release of bFGF into the culture medium [McNeil et al., 1989]. Sublethal injury to EC has been documented to occur in situ, presumably due to hemodynamic forces including shear stress and turbulent flow [Davies, 1988]. The injury is evidenced in a higher labeling index of EC at vessel bifurcations vs. unbranched regions [Wright, 1968].

Taken together, these observations have led to the proposal that bFGF may function as a "wound hormone," both in the routine maintenance of tissue integrity and during repair after injury [Muthukrihnan et al., 1991]. In support of this concept, bFGF expression has been shown to be elevated at the site of injury in several experimental models. There is a marked increase in bFGF immunoreactivity following mechanical lesion to the cerebral cortex [Finklestein et al., 1988]. Similarly, isoproterenol-induced cardiomyocyte injury leads to an increase in both bFGF mRNA and protein within 24 h [Padua and Kardami, 1993]. Finally, optic nerve crush in the mouse results in a dramatic increase in bFGF staining in the photoreceptor layer of the retina [Kostyk et al., 1994].

The work of Florkiewicz and his colleagues [Florkiewicz et al., 1991] has provided some insight into the molecular regulation of the bFGF gene. Primer extension analysis suggests that a single transcription initiation site is utilized to generate multiple bFGF transcripts. Analysis of the bFGF gene promoter has revealed the presence of five GC boxes (binding sites for the transcription factor (SP-1) and one potential AP-1 transcription factor binding site, a finding consistent with the stimulation of bFGF mRNA levels by phorbol esters [Murphy et al., 1988]. However, the bFGF promoter does not contain the consensus CAAT or TATA box motifs. In addition, functional analysis of the bFGF promoter suggests the presence of two negative regulatory domains, which may account for the low level of transcription measured in unstimulated cells [Goldsmith et al., 1991]. Together these observations suggest that bFGF gene expression is controlled at the transcriptional level by the interaction of multiple regulatory factors.

A number of factors have been reported to modulate bFGF mRNA levels, including serum [Murphy et al., 1988; Winkles and Gay, 1991], thrombin [Weich et al., 1991], IL-1 [Gay and Winkles, 1991], and TGF-β1 [Cook et al., 1990; Winkles and Gay, 1991]. bFGF has also been shown to induce its own expression in capillary endothelial cells [Weich et al., 1991]. In addition, bFGF gene expression was found to be inversely proportional to cell density, suggesting that it may be controlled by cell-cell contact or by factors present in the extracellular matrix [Murphy et al., 1988].

Injury appears to be both a likely mode for bFGF release and a stimulator of bFGF expression. However, little is known about the regulation of bFGF expression and about the role that it may play in wound healing. To address these questions, we have utilized an in vitro model of cell injury in which scraped EC have been shown to release bFGF. We found that scraping EC led to the release of up to 80% of cell-associated bFGF and induced a significant (4- to 10-fold) increase in steady state bFGF mRNA levels. The inclusion of specific antagonists revealed that proteases and growth factors released and/or activated by injury contribute to the regulation of bFGF mRNA and protein following bFGF release and may mediate the recovery of the injured cells. Furthermore, we found that transcriptional activation of the bFGF gene accounts for the increase in steady state bFGF mRNA.

MATERIALS AND METHODS

Cell Cultures

Bovine aortic endothelial cells (BAE) used in this study were obtained and maintained according to previously described methods [Gospodarowicz et al., 1976] and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS) supplemented with penicillin (10,000 U/ml) and streptomycin C (10,000 μg/ml; Irvine Scientific, Santa Ana, CA). Cells between passages 7 and 25 were used. The cells were demonstrated to be mycoplasma-free prior to use.

Sublethal Cell Injury

To induce the release of bFGF, BAE grown to 80–90% confluence in 150-mm culture dishes were scraped from the plastic substratum with a rubber policeman. Immediately following scraping, the cells were triturated with a 10-ml pipette to create a single cell suspension and then allowed to recover and reattach to the same dish in the original medium.

To demonstrate that scraping creates transient breaks in the cells' plasma membranes, FITC-labeled dextran, a molecular marker that does not penetrate the plasma membrane of intact cells, was included. BAE were scraped in the presence of 10 mg/ml of FITC-labeled dextran (average molecular weight of 10,000; Sigma, St. Louis, MO). After 1 h cells were washed several times with PBS and then examined by fluorescence microscopy. Control unscraped cells were also incubated with 10 mg/ml of FITC-labeled dextran for 1 h.

In other experiments, specific antagonists of proteases and growth factors were added to the cells prior to scraping. The antagonists employed included PAI-1 (American Diagnostica, Inc., Greenwich, CT), aprotinin (CalBiochem, La Jolla, CA), heparin (Hepar Industries, Inc., Franklin, OH), cycloheximide, a-amanitin, AT III, hirudin (all from Sigma), suramin (Miles, Inc, Naperville, IL) egg white trypsin inhibitor (Boehringer Mannheim, Indianapolis, IN), and neutralizing antibodies against TGF-β1 (R&D Systems, Minneapolis, MN) and against human recombinant bFGF (kindly provided by Dr. Michael Klagsbrun, Children's Hospital, Boston).

Northern Blot Analysis

Total RNA was prepared using the guanidine isothiocyanate method [Chirgwin et al., 1979]. Total RNA (10–30 μg) was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde in 5 × morpholinopropano sulfonic acid. The gel was stained with ethidium bromide to visualize rRNA, and the RNA was transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH) by capillary action overnight in 10 × SSPE (1 × SSPE = 0.15 M NaCl/0.01 M $NaH_2PO_4 \cdot H_2O$/0.001 M EDTA – Na_2, pH 7.4). Following transfer, the nitrocellulose paper was baked at 80°C under vacuum for 2 h and then prehybridized overnight at 65°C in a buffer containing 6 × SSPE, 6 × Denhardt's, 0.5% SDS, and 100 μg/ml salmon sperm DNA. cDNA probes were denatured by boiling and added to the pre-hybridization solution. Following hybridization overnight at 65°C, the blots were washed at 50°C in 0.5 × SSPE/0.1% SDS until the background radioactivity was removed.

The bFGF cDNA probe was a 1.0 kb EcoRI-NcoI fragment of the bovine bFGF cDNA clone pJJ11-1 (kindly provided by Drs. J. Abraham and J. Fiddes, Scios-Nova, Mountain View, CA). The cDNA probes were labeled with [a-^{32}P] dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA) using a random hexamer priming kit (Boehringer Mannheim).

To normalize for RNA loading, the blots were probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [Fort et al., 1985] and quantified in a phosphoimager (Molecular Dynamics, Sunnyvale, CA) using Image Quant software (Molecular Dynamics). The level of GAPDH mRNA was found to remain constant for up to 24 h following scraping, as indicated by the consistency between GAPDH mRNA level and the rRNA level. The relative intensity of each bFGF signal was expressed as a fraction of the corresponding GAPDH signal; the bFGF/GAPDH in the untreated control was set to 1.

BAE Proliferation

For the analysis of BAE proliferation following injury, cells were plated at 5,000 cells/cm^2 in 6-well culture plates and grown to 70% confluence, scraped with a rubber policeman and triturated with a 1 ml pipette to create a single cell suspension. The cells were allowed to recover and reattach in the same medium for 2 h. In some experiments, cells were pretreated with specific antagonists of proteases and growth factors for 1 h prior to scraping. Unattached cells and medium were removed and fresh DMEM/10% FCS was applied and cell growth was monitored for 3 days using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Determination of bFGF Protein Levels

The amount of bFGF protein in the conditioned media and lysates of scraped and control BAE was determined using a bFGF immunoassay (provided by R&D Systems) according to the manufacturer's instructions. In this assay, bFGF in the test sample is sandwiched between a monoclonal antibody against human recombinant bFGF coated on the microtiter plate, and a second polyclonal antibody against bFGF conju-

gated to horseradish peroxidase. Color is developed by addition of hydrogen peroxide and chromogen tetramethylbenzidine and the intensity measured at 450 nm. The assay is sensitive to 10 pg/ml and has inter- and intra-assay variation of 6.4 and 4.6–7.1%, respectively.

To analyze released bFGF, heparin was added to the media to a final concentration of 10 μg/ml to displace bFGF from cell surface and extracellular matrix heparan sulfate. The media were collected, clarified by centrifugation, and bFGF levels measured by immunoassay. The attached cells were removed by trypsinization and an aliquot withdrawn to determine cell number for the purpose of normalizing bFGF protein levels. For lysate preparation, the cells were pelleted by centrifugation and washed three times in the following solution: PBS, 2 mM phenylmethanesulfonyl-fluoride, 2 mM 4-(2-aminoethyl)-benzenesulfonyl-fluoride, HCl, 2 mM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The cell pellet was resuspended in a solution containing 10 mM Tris-HCl (pH 7.4), 1% NP-40, 0.1% Chaps, 2 M NaCl, 10 μg/ml heparin with protease inhibitors (at the same concentration as the PBS wash), and pushed through a syringe fitted with 30-gauge needle. The homogenate was then clarified at 14,000 rpm for 5 min and diluted to a final concentration of 0.4 M NaCl for assay. Normal serum has a bFGF level of 5–6 pg/ml [Ii et al., 1993], which is undetectable in this assay.

Nuclear Run-On Analysis

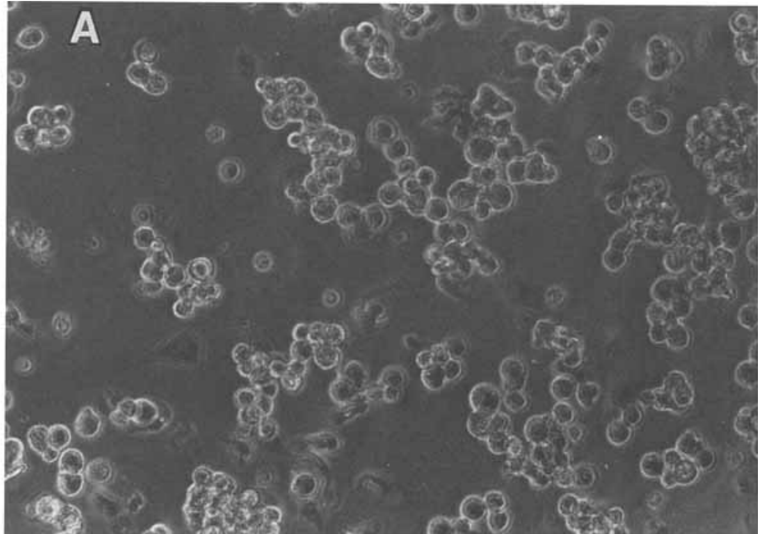
The rate of transcription of the bFGF gene was measured by a modification of a nuclear run-on assay described previously [Kavanaugh et al., 1988]. To prepare nuclei, BAE were lysed in ice-cold buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40 (20 ml lysis buffer/2.5–3 × 10^7 cells). The cells were homogenized with a Dounce (70 strokes) and centrifuged at 1,200 rpm for 5 min at 4°C. The nuclear pellet was washed with lysis buffer containing 0.025% NP-40, recentrifuged, and resuspended in buffer containing 20 mM Tris (pH 8.1), 75 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 50% (v/v) glycerol to a final volume of 200 μl. The resuspended nuclei were flash-frozen in liquid nitrogen and stored at −80°C until use.

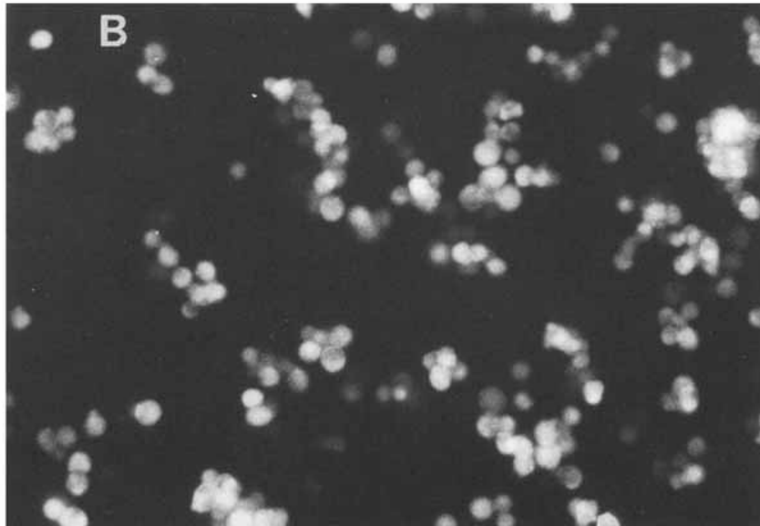
The reaction mixture for the nuclear run-on contained 200 μl of nuclei, 250 μCi of 3,000 Ci/mmol ^{32}P-UTP (NEG-007 H; New England Nuclear, Boston, MA), 55 μl of 5 × transcription buffer (100 mM Tris, pH 8.0, 500 mM KCl, 25 mM $MgCl_2$, 10 mM DTT, 500 μM each of ATP, GTP, and CTP), and 2–5 μl of RNAsin (40,000 U/ml; Promega, Madison, WI). This mixture was incubated at 20°C for 30 min and centrifuged to collect the nuclear pellet, which was resuspended in 200 μl 1 × transcription buffer. The run-on reaction was terminated by adding 25 U of RNase-free DNase I (Boehringer Mannheim) and 50 μg of yeast tRNA (Boehringer Mannheim), and incubating at 37°C for 15 min. The mixture was then treated with 25 μl of 10% SDS and 20 μl of 10 mg/ml proteinase K (United States Biochemical, Cleveland, OH) and incubated at 37°C for 30 min. RNA was isolated and resuspended to equal counts/min/ml in hybridization buffer (1 × 10^7 CPM/ml) containing 40% formamide, 4 × SSPE, 5 mM EDTA, 0.4% SDS, 1 × Denhardt's solution, and 100 μg/ml yeast tRNA. Hybridization to 1 μg denatured purified bFGF cDNA inserts dot-blotted on nitrocellulose filters was performed at 40°C for 3 days. The filters were washed twice in 0.3 M NaCl/2 mM EDTA/10 mM Tris (pH 7.5)/0.4% SDS at 45°C for 30 min, and twice in 0.3 M NaCl/2 mM EDTA/10 mM Tris (pH 7.5) briefly at room temperature to remove SDS. The filters were then treated with 10 μg/ml RNase A (Boehringer Mannheim)/0.3 mM NaCl/2 mM EDTA/10 mM Tris (7.5) at 37°C for 1 h. Finally, the filters were washed twice in 0.3 M NaCl/2 mM EDTA/10 mM Tris (pH 7.5)/0.1% SDS at 45°C for 1 h, air-dried and exposed to X-ray film at −70°C for 1 week.

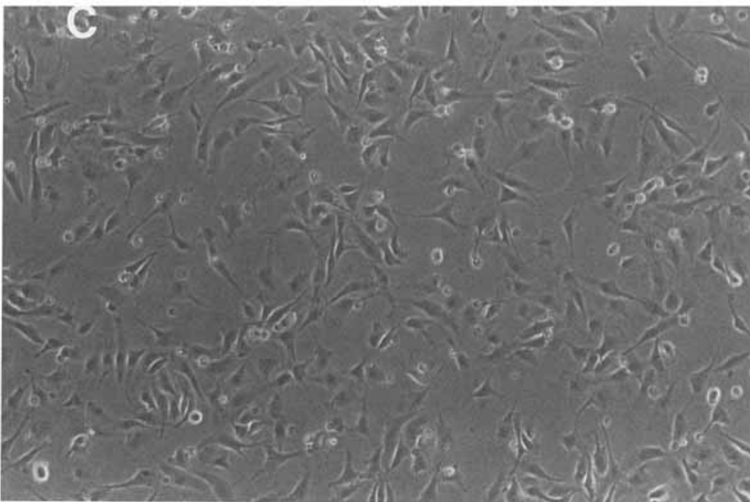
The bFGF cDNA insert was a 1.045 kb EcoRI-NcoI fragment of pJJII-1 bFGF plasmid DNA from Dr. Judith Abraham (Scios-Nova). Genomic DNA from BAE (0.2 μg/dot) was used to standardize the transcriptional level under various conditions.

Assessment of mRNA Stability

Unstimulated levels of bFGF mRNA are low. In order to examine the effect of scraping on bFGF mRNA stability it was necessary to induce a pool of bFGF mRNA. To increase bFGF mRNA, BAE were treated with 0.5 ng/ml TGF-β1 (Oncogen, Seattle, WA) for 2 h. The cells were rinsed with DMEM/1% BSA to remove TGF-β1 and were then fed with DMEM/1% BSA containing 5 μg/ml actinomycin D (Fluka, Ronkokoma, NY) to block further transcription. To determine the effect of scraping on bFGF mRNA







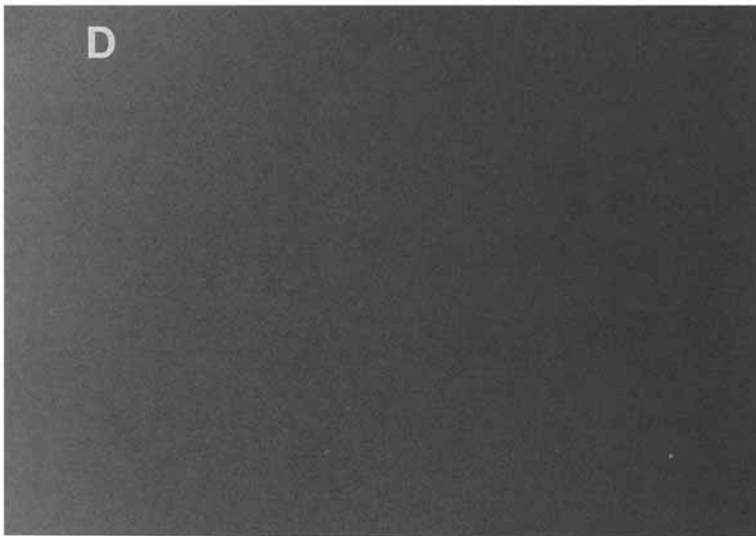


Fig. 1. Induction of sublethal injury by scraping. Subconfluent cultures of BAE were scraped in the presence of FITC-labeled dextran (MW 10,000), a marker excluded by intact cells. Paired phase (**A**) and fluorescent (**B**) micrographs reveal >95% of the cells were labeled with fluorescein by scraping. The unscraped control cells were not labeled by the dye during the same time period (**A** and **D**).

stability one group of cells was then scraped and allowed to recover, while a second group was left untreated. At various times total RNA was isolated from cells and analyzed for bFGF mRNA levels by Northern analysis.

RESULTS

Levels of bFGF mRNA and Protein in BAE Following Sublethal Injury

McNeil and his co-workers have shown that scraping attached cells creates transient breaks in the plasma membranes, leading to a flux of molecules between the cells and their environment [McNeil and Ito, 1989]. In agreement with these findings, we observed that the inclusion during scraping of FITC-labeled dextran, a marker excluded by the intact cells, led to labeling of more than 95% of the scraped cells (Fig. 1A,B), whereas unscraped cells were not labeled by the dye during the same time period (Fig. 1C,D).

Sublethal injury of BAE has been reported to release bFGF from cells [Muthukrihnan et al., 1991]. We were interested in quantifying this release and in determining if and how the cells replenish bFGF lost as a result of sublethal injury. bFGF was measured in the conditioned media and cell extracts of BAE prior to scraping and at various times following scrape injury. There was no bFGF in the media of unscraped BAE (Fig. 2A). Scraping BAE led to the release of approximately 80% of the cell-associated bFGF into the media. The magnitude of bFGF release was dependent on the degree of cell confluence at the time of scraping. Whereas 60–80% of the bFGF was released when subconfluent cells were scraped, a more confluent culture released only 30–40% of total bFGF upon scraping (data not shown). The lower level of bFGF released by the more confluent cells is likely to be due to the fact that scraping confluent cells tends to release them as a sheet, minimizing membrane tearing. Following the initial loss, cell-associated bFGF was quickly restored; 6 h after scraping bFGF was returned to basal levels (Fig. 2B). The level of cell associated bFGF did not exceed the initial levels even at later time points.

Unscraped BAE grown to 80–90% confluence had low levels of bFGF mRNA (Fig. 3A, lane 1). Following scraping, the levels of two bFGF transcripts, 7.0 and 4.0 kb, were elevated. An 8- to 10-fold increase in steady state bFGF mRNA level was observed 2 h following bFGF release (lane 3). (Over multiple experiments, the increase varied between 4- and 10-fold.) The transcript levels declined to baseline by 6 h following scraping (lane 4) and then increased again at 24 h (lane 5). This pattern of bFGF mRNA expression, a reduction at 6 h and increase at 24 h, was reproducible and was observed in 4 separate experiments. The level of the 7.0 kb message was greater than that of the 4.0 kb message. Though the significance of this difference is not clear, it has been speculated that variation in the 3′-untranslated region of the two transcripts may contribute to their differential regu-

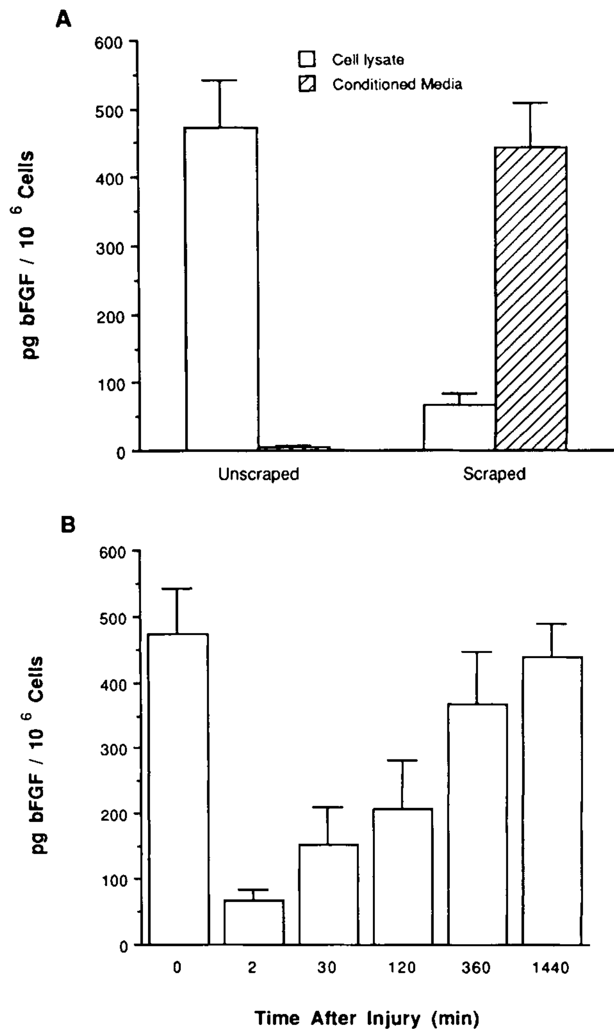


Fig. 2. Release of bFGF by scraping. BAE were scraped in serum-containing medium. An aliquot of the cells was taken to determine cell number. Cell lysates and conditioned media were collected and assayed for bFGF protein by ELISA. (A) At the time of injury and (B) over a 24 h time period following injury. Values represent means of triplicate samples ±SD. Sublethal injury resulted in the release of >80% of the cell-associated bFGF. By 6 h following injury, intracellular bFGF level was restored to the levels in unscraped cells.

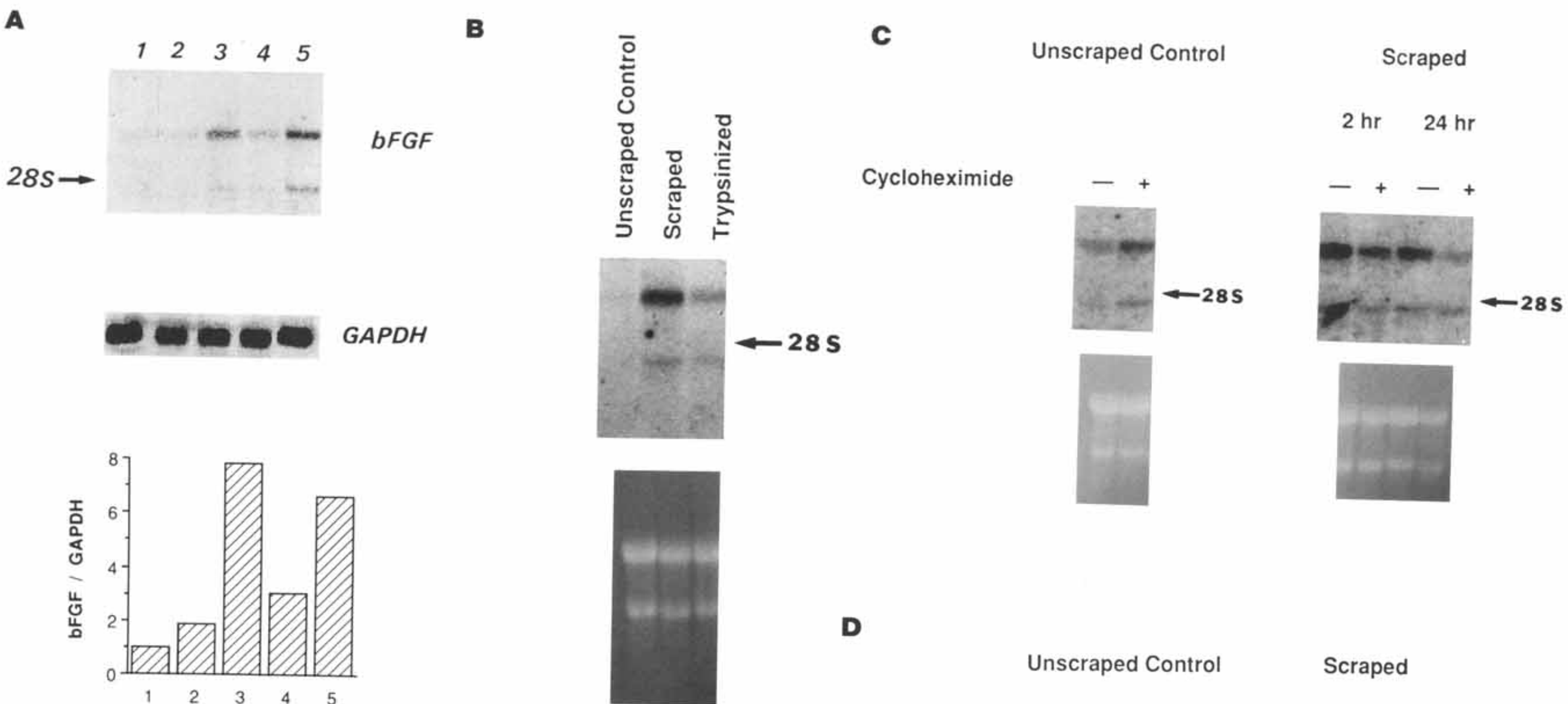


Fig. 3. The effect of scrape-injury on the level of bFGF mRNA in BAE. **A:** BAE were scraped in serum-containing medium. Total RNA (20 μg) from unscraped cells and cells at various time points following scraping was analyzed by Northern blot analysis for expression of bFGF mRNA (top panel). As a control for loading, the blot was probed for GAPDH expression. The bottom panel illustrates the quantification of the 7.0 kb bFGF transcript at times following scraping. **Lanes 1, 2, 3, 4,** and **5** represent 0, 0.5, 2, 6, and 24 h following scraping. **B:** One set of unscraped cells was incubated at 37°C for 2 h. A second set of cells was scraped in serum-containing medium, allowed to recover for 2 h and the number of cells that reattached at 2 h was determined. A third set of cells was trypsinized and then replated at a density to match that of the second set of cells. At 2 h after plating total RNA was isolated. **C:** BAE were scraped in serum-containing medium in the presence and absence of 10 μg/ml cycloheximide. At 2 and 24 h following scraping, total RNA was isolated. **D:** BAE were scraped in serum-containing medium in the absence and presence of 10^{-8}, 10^{-7}, and 10^{-6} M α-amanitin; 2 h after scraping total RNA was isolated. For B–D, total RNA was isolated and ethidium bromide staining of the RNA gel confirmed equal loading.

lation [Bost et al., 1992]. Acidic FGF (FGF-1) transcripts were undetectable in the control and scraped cells (data not shown). Taken together, the mRNA and protein data indicate that BAE have the ability to rapidly replenish bFGF lost as a result of sublethal injury.

bFGF gene expression has been shown to increase following replating of cells at low density [Murphy et al., 1988]. To eliminate the possibility that cell plating and/or density might account for the observed increase in bFGF mRNA, we compared the expression of bFGF mRNA in BAE that had been scraped and replated to cells that were trypsinized and replated at a similar density. Trypsinization and replating led to an elevation in bFGF mRNA level (Fig. 3B). However, the increase was much less than that observed following scraping, indicating that the increase in bFGF mRNA was not due to replating or density alone.

To characterize the mechanism of the increase in bFGF mRNA, the effects of cycloheximide, a protein synthesis inhibitor, and α-amanitin, an inhibitor of RNA polymerase II, were assessed. The inclusion of cycloheximide led to a significant but not total reduction in the scrape-induced increase in bFGF mRNA, indicating some dependence on protein synthesis (Fig. 3C). In addition, the effect of cycloheximide on the

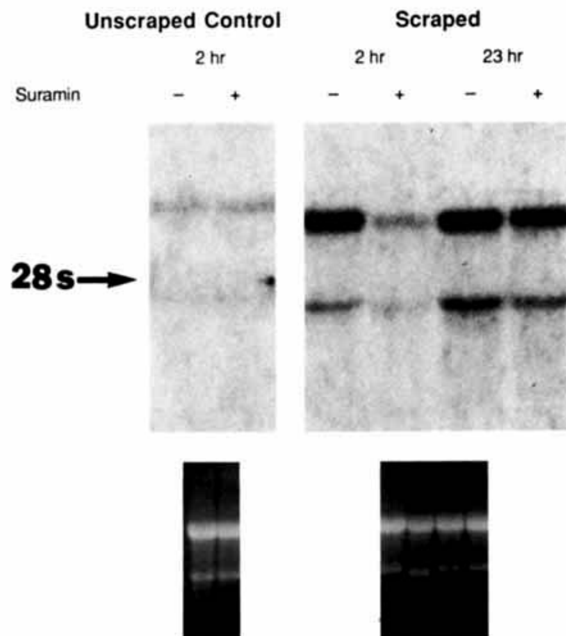


Fig. 4. Effect of suramin on the level of bFGF mRNA following scraping. BAE were scraped in serum-containing medium in the absence and presence of 0.5 mM suramin. At 2 and 23 h following scraping, total RNA was isolated and analyzed by Northern blotting for expression of bFGF mRNA.

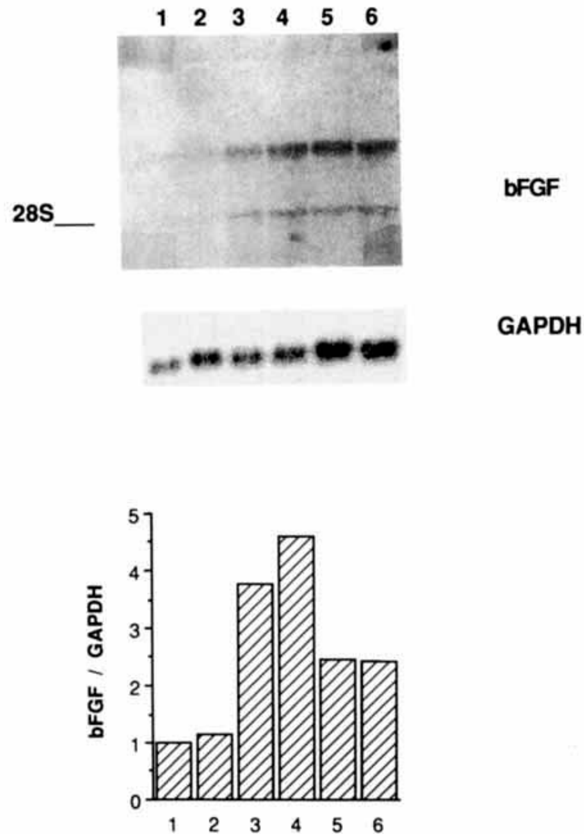


Fig. 5. Effect of anti-bFGF on the level of bFGF mRNA in BAE following injury-induced bFGF release. BAE were scraped in serum-containing medium in the presence and absence of neutralizing antibodies against bFGF (27 μg/ml). At 4 and 24 h following scraping, total RNA was isolated and 30 μg was analyzed by Northern blotting for expression of bFGF mRNA (**top**). As a control for sample loading, the blot was probed for GAPDH expression (**middle**). The **lower panel** represents the quantification of the 7.0 kb bFGF transcript level following various treatments. **Lanes 1, 3,** and **5** represent time points of 0, 4, and 24 h following injury in the absence of antibodies. **Lanes 2, 4,** and **6** are the same time points in the presence of antibodies. Neutralizing antibodies against bFGF did not significantly influence the increase in bFGF mRNA level following scraping.

TABLE I. Neutralization of bFGF's Biological Activity With Neutralizing Antibodies Against bFGF*

	BAE cell number	
−bFGF	75,200 ± 4,534	63,697 ± 2,535
+bFGF	99,870 ± 7,321	59,963 ± 5,933

*BAE were plated at 10,000/well in 24-well plates and allowed to attach for 24 h in DMEM/10% FCS. The medium in each well was replaced the following day with DMEM plus 2% FCS with either no additions or 5 ng/ml human recombinant bFGF with or without 27 μg/ml of bFGF neutralizing antibodies. Cells were allowed to grow for 3 days, then trypsinized and cell number determined with a Coulter counter. Each value represents the mean of triplicate samples ± standard deviations.

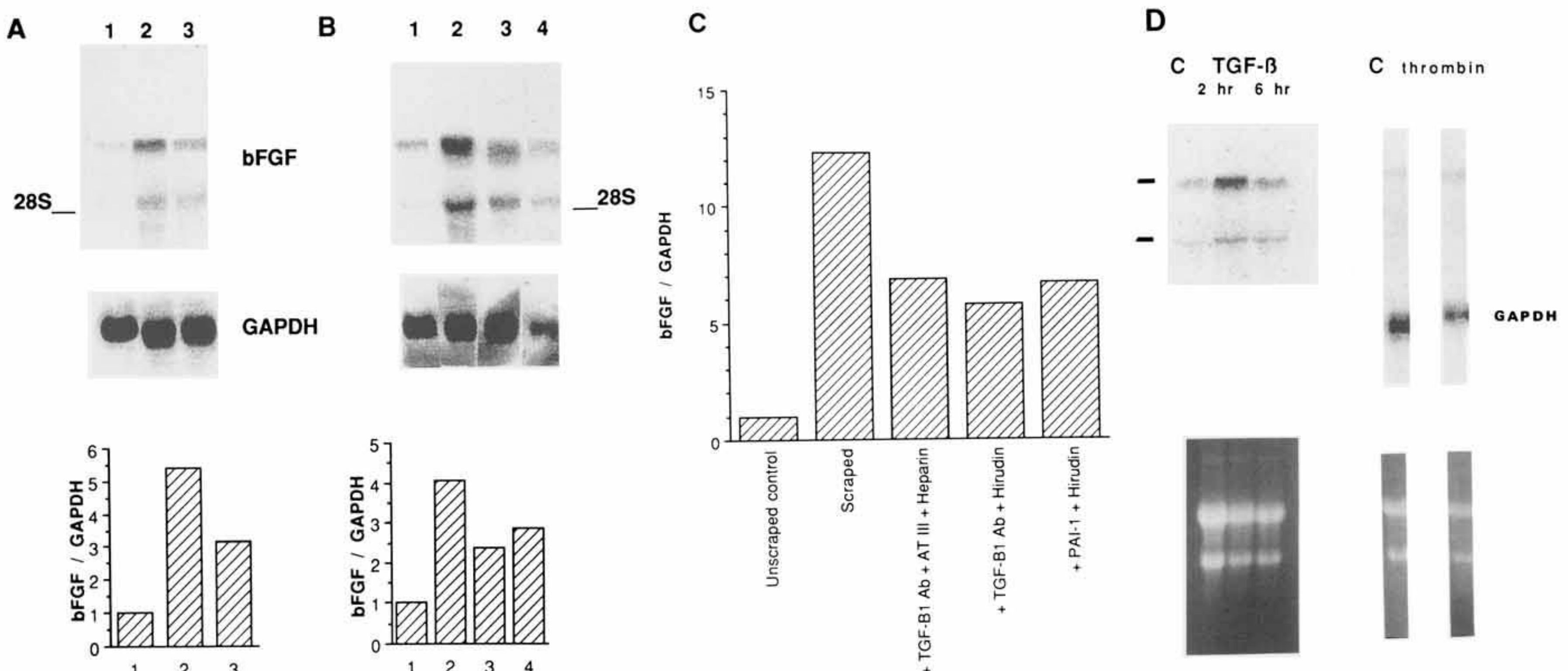


Fig. 6. Effect of inhibiting TGF-β1 (**Panel A**), plasminogen activator, and thrombin (**Panel B**) on the level bFGF mRNA in BAE following injury. BAE were scraped in serum-containing medium without inhibitor (**lane 2** of **A** and **B**), 4 μg/ml TGF-β1 neutralizing antibodies (**lane 3** of **A**), 0.5 U/ml AT III plus 100 μg/ml heparin (**lane 3** of **B**), and 2 U/ml PAI-I (**lane 4** of **B**). **Lane 1** of both A and B is RNA from unscraped controls cells. At 2 h following scraping, total RNA was isolated and 15 μg was analyzed by Northern blot analysis for expression of bFGF mRNA (**top**). As a control for sample loading, the blot was probed for GAPDH mRNA level (**middle**). Lower panel represents the quantification of the 7.0 kb bFGF transcript level following various treatments. Inhibition of TGF-β, plasminogen activator, and thrombin each led to a 50% reduction in bFGF mRNA. **C:** BAE were scraped in serum-containing medium without inhibitor or in the presence of combination of various inhibitors as indicated (concentrations are indicated in the legend of parts A and B). Hirudin (1 U/ml) was also used. Quantification of the 7.0 kb bFGF transcript level following various treatments is shown. The simultaneous addition of antagonists to both TGF-β1 and thrombin led to an approximately 50% reduction in the scrape-induced increase in bFGF mRNA. **D:** BAE were incubated in the presence of TGF-β (0.5 ng/ml) for 2 and 6 h (**left**) or thrombin (5 U/ml) for 2 h. Cells were harvested and total RNA isolated and probed for the expression of bFGF mRNA.

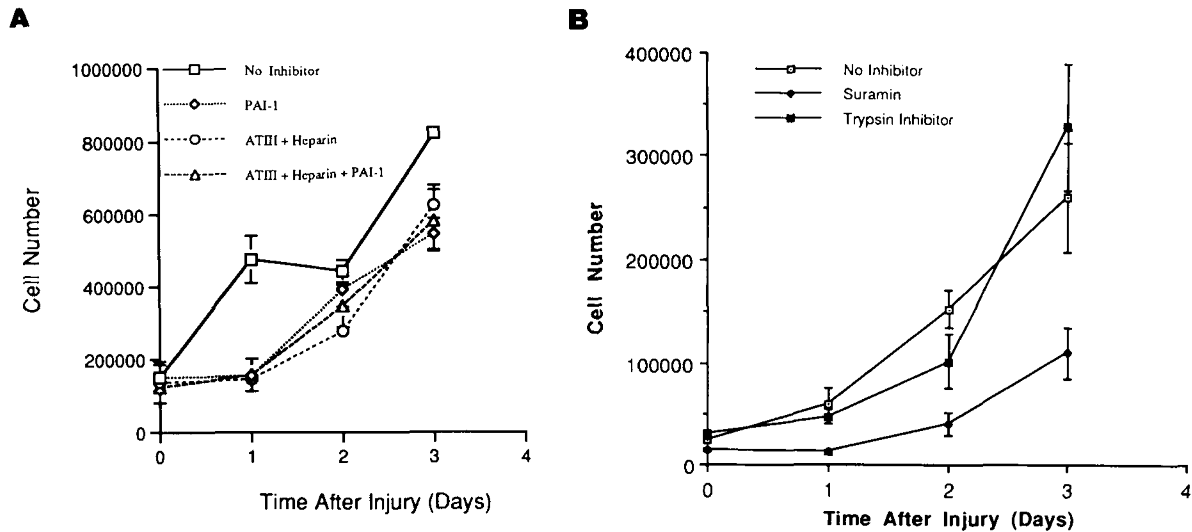


Fig. 7. Effect of inhibiting TGF-β1, plasminogen activator, and thrombin on growth of BAE following on scrape-induced injury. BAE were pre-incubated for 1 h at 37°C in the absence and presence of (A) PAI-I or anti-thrombin III plus heparin, or a combination of the two (at concentrations indicated above), or (B) 50 μg/ml egg white trypsin inhibitor or 0.05 mM suramin before being scraped in serum-containing medium. Two hr following scraping, unattached cells were removed, and the attached cells were then fed with fresh DMEM/10% fetal calf serum without inhibitors, and their growth monitored for 3 days. Cells recovered and proliferated in the absence of inhibitors. Suramin, PAI-I, AT III plus heparin, or a combination of PAI-I and AT III plus heparin blocked the recovery and proliferation of the scraped cells. The specificity of the effect is demonstrated by the lack of inhibition of egg white trypsin inhibitor.

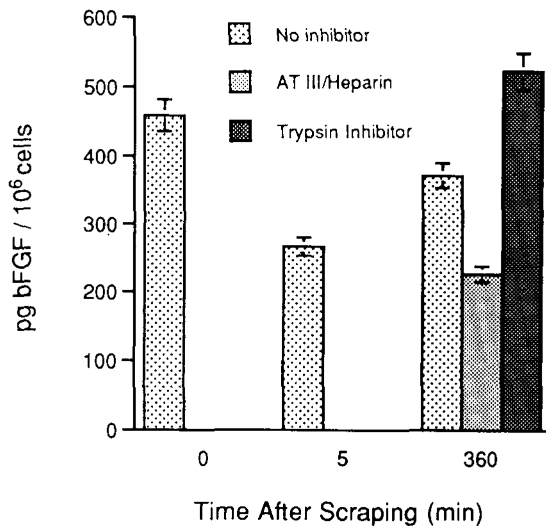


Fig. 8. Effect of AT III plus heparin, suramin, and trypsin inhibitor on the recovery of bFGF protein following scraping. BAE were scraped in serum-containing medium in the absence and presence AT III (0.5 U/ml) plus heparin (100 μg/ml) or egg white trypsin inhibitor (50 μg/ml). Before injury and at 5 min and 6 h after scraping, cell lysates were collected and assayed for bFGF protein level by ELISA. Values represent means of triplicate samples ± SD.

increase in bFGF mRNA observed at 24 h after scraping was significantly greater than its effect on the increase at 2 h. Cycloheximide treatment (2 h) of unscraped cells resulted in an increase in bFGF mRNA, an observation consistent with the suggestion that labile repressors [Florkiewicz et al., 1991] and/or mRNA stability play a role in the regulation of bFGF mRNA levels. The difference in the effect of cycloheximide on bFGF mRNA in scraped and unscraped cells suggests that different mechanisms are involved in maintaining baseline bFGF levels and restoring bFGF after scraped-induced release. Inclusion of α-amanitin (10^{-8} M) almost completely blocked the elevation in bFGF mRNA level following scraping (Fig. 3D), indicating that transcription is essential for the injury-induced increase in steady state level of bFGF mRNA.

Mediators of Increased bFGF mRNA Following Injury

We were interested in identifying the factors involved in bFGF gene expression following scrape-induced bFGF release. We speculated that at least a portion of the increase in bFGF mRNA might be mediated by soluble factor(s) released and/or activated by scraping. To test this hypothesis, we examined the effect of suramin on bFGF mRNA level following injury. Suramin, a polyanionic compound which has been shown to interfere with the interaction between many growth factors and their cell surface receptors [Coffey et al., 1987; Hosang, 1985; Pollack and Richard, 1990], suppressed the increases in bFGF mRNA at 2 h following scraping by ap-

proximately 70% (Fig. 4). (Over multiple experiments the inhibitory effect of suramin varied between 70 and 90%.) The increased bFGF mRNA at 24 h following scraping was not influenced by suramin, again suggesting a difference between the mechanisms leading to the increases at 2 and 24 h.

Since bFGF has been shown to induce its own gene expression [Weich et al., 1991], we postulated that the bFGF released from scraped cells might act in an autocrine fashion to induce its own mRNA expression. To examine this possibility, neutralizing antibodies against bFGF were included at the time of scraping (Fig. 5). However, the antibodies did not significantly influence mRNA levels at 4 h (lanes 3 and 4) and 24 h (lanes 5 and 6) following scraping. Addition of antibodies 5 min after scraping also had no effect (data not shown). Pre-treatment of BAE with neutralizing anti-bFGF antibodies for 1 h prior to scraping, suppressed the increase in bFGF mRNA level by approximately 15% (data not shown). The efficacy of the antisera in neutralizing the biologic activity of bFGF was confirmed in a parallel experiment in which the antisera completely blocked the growth-promoting activity of 5 ng/ml of recombinant bFGF on BAE (Table I). These observations suggest that the autocrine action of bFGF accounts for only a minor portion of the injury-induced increase in bFGF mRNA level. This is further supported by the finding that exogenous bFGF (5 ng/ml) induced only a small increase (about 2-fold) in bFGF mRNA level (data not shown).

Role of TGF-β1, Plasminogen Activator, and Thrombin in the Regulation of bFGF mRNA

We next focused our attention on agents known to be involved in wound healing. One candidate was transforming growth factor-beta (TGF-β1), whose versatile role in wound healing has been well documented [for review see Roberts and Sporn, 1990]. EC, like many other cells in culture, produce TGF-β1 in a latent form [Antonelli-Orlidge et al., 1989] and we speculated that scraping BAE might release proteases which would activate the latent TGF-β1 [Lyons et al., 1988]. Furthermore, TGF-β has been reported to increase bFGF mRNA [Cook et al., 1990; Sternfeld et al., 1988; Winkles and Gay, 1991] and we have found that TGF-β1 (0.5 ng/ml) led to a 13-fold increase in steady state levels of bFGF mRNA in BAE (Fig. 6D; see also Fig. 10). Inclusion of neutralizing antibodies against TGF-β1 at the time of injury suppressed the increase in bFGF mRNA by approximately 40% (Fig. 6A, lanes 2 and 3). The addition, at the time of scraping, of plasminogen activator inhibitor-1 suppressed bFGF mRNA levels by about 25%, although values across experiments ranged from 25–50% inhibition (Fig. 6B, compare lanes 2 and 4).

Another molecule implicated in wound healing, both as a growth regulator [Shuman, 1986] and in the clotting cascade, is thrombin. The addition of thrombin (5 U/ml) led to a 2–3-fold increase in the steady state levels of bFGF mRNA in BAE (Fig. 6D). To assess whether thrombin was involved in injury-induced bFGF mRNA expression, the thrombin neutralizing combination of AT-III and heparin was added to the BAE at the time of scraping; the increase in bFGF mRNA was suppressed by approximately 50% (Fig. 6B, compare lanes 2 and 3).

Since TGF-β and thrombin each appeared to account for 50% of the increase in bFGF mRNA, we examined the possibility that the combination of TGF-β1 and thrombin might account for all of the injury-induced increase bFGF mRNA. The simultaneous addition of anti-TGF-β antibodies along with AT III and heparin led only to a 50% reduction in scrape-induced bFGF mRNA (Fig. 6C). Similarly, the combination of hirudin, another thrombin inhibitor, with TGF-β antisera or PAI-1 led to a 40–50% reduction in bFGF mRNA levels. These results suggest that these agents act via overlapping or redundant pathways to increase bFGF mRNA and indicate that other factor(s) are involved in the regulation of bFGF mRNA level following scrape injury.

BAE Proliferation Following Scraping

We next investigated whether antagonists which blocked the increases in bFGF mRNA, influenced the recovery and proliferation of BAE following scraping. The effect of a panel of growth factor and protease antagonists on the proliferation of scraped cells was studied. Following scraping, 40–60% of the BAE reattached. Antagonists of plasminogen activator and thrombin, added singly or in combination for 2 h following scraping, suppressed the proliferation of the reattached cells at 24 h when compared to cells scraped in the absence of additions (Fig. 7A). The less specific growth factor inhibitor suramin similarly suppressed cell recovery (Fig. 7B). To demonstrate that the growth suppression

was not due to toxicity, the cells were allowed to grow for 2 additional days; normal growth resumed. The effects of these antagonists were specific as the inclusion of another protease inhibitor, egg white trypsin inhibitor, did not block the growth of the reattached BAE (Fig. 7B).

To confirm that the reduction in bFGF mRNA observed following treatment with these antagonists was reflected in lowered bFGF protein, the effect of AT III and heparin on the recovery of bFGF protein following scraping was assessed. Whereas cells scraped in the absence of any antagonist replaced their intracellular bFGF within 6 h after scraping, AT III plus heparin reduced the level of bFGF in BAE at 6 h after scraping by approximately 40% (Fig. 8). This value correlates well with the 50% inhibition in mRNA level affected by AT III and heparin. The specificity of these effects is evidenced by the fact that the trypsin inhibitor, which had no effect on the proliferation of cells following scraping, also did not suppress bFGF levels. Trypsin inhibitor did, however, lead to a significant increase in bFGF levels. This effect may be due to blocking the activity of trypsin-like enzymes in serum, which would otherwise degrade bFGF. Thus, agents which blocked the replenishment of cell associated bFGF also interfered with cell recovery and proliferation.

Transcriptional Activity of bFGF Gene Following Scraping

The steady state level of any mRNA is determined by a balance between the rates of transcription and mRNA degradation. To determine the relative contribution of these processes to the steady state level of bFGF mRNA following sublethal injury, BAE were injured in a nonlethal manner by scraping and were allowed to recover. At various times following scraping, the cells were lysed, nuclei isolated, and the transcriptional activity of bFGF gene was determined by nuclear run-on, with an equal number of nuclei used for each time point. The rate of transcription in the unstimulated cells was very low (Fig. 9), an observation consistent with the low level of bFGF seen in unscraped cells by Northern analysis. At 2 h following injury, the transcription rate of the bFGF gene was increased. This was not due to a general increase as the transcriptional rate of the endothelin gene was decreased at the same time point.

Effect of Sublethal Injury on Stability of bFGF mRNA

We also investigated whether changes in mRNA stability were contributing to the increase in steady state level of bFGF mRNA following its scrape-induced release. Unstimulated BAE have a low level of bFGF mRNA and therefore provide poor starting material for measuring bFGF mRNA stability (Fig. 3A). To overcome this problem, BAE were stimulated with TGF-β to increase bFGF mRNA. The TGF-β-treated cells were then used to determine whether sublethal injury influences bFGF mRNA stability. BAE were incubated with 0.5 ng/ml TGF-β1 for 2 h, followed by extensive rinsing to remove the growth factor. The cells were then fed with fresh media containing actinomycin D to block further transcription. One group of cells was scraped while a second set was left unscraped. A third group of cells was scraped without prior actinomycin D treatment. At various times after injury, total RNA was isolated from cells of various treatment groups and examined by Northern analysis. As seen in the left panel of Figure 10, both the 7.0 kb and 4.0 kb transcripts were rapidly degraded in unscraped BAE, with an apparent half-life of less than 1 h. There appears to be two distinct populations of bFGF mRNA in these cells; while a majority of the transcripts were rapidly degraded within 1 h following the inhibition of transcription, a subpopulation of mRNA appears to be relatively stable over the 6 h of the time course. Scraping the actinomycin D-treated cells did not appear to influence the stability of the bFGF mRNA (middle panel of Fig. 10). Interestingly, the level of bFGF mRNA was further increased in scraped cells with no prior cycloneximide D (right panel of Fig. 3), suggesting that scrape-injury (or the release of bFGF) induces the synthesis of a labile regulatory factor that plays a role in the upregulation of bFGF mRNA.

DISCUSSION

The concept of sublethal injury as a physiologically relevant mode of bFGF release is supported by several lines of experimental evidence [Gajdusek and Carbon, 1989; McNeil et al., 1989; Witte et al., 1989]. However, it was not known if, how, or when injured cells "replenish" lost bFGF. To address these issues, we examined the levels of bFGF protein and mRNA in BAE following its release secondary to nonlethal injury. As

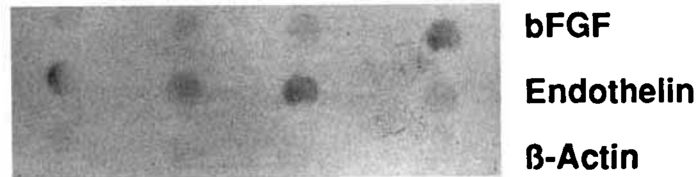


Fig. 9. The effect of scraping on transcriptional activity of bFGF gene. BAE (80–90% confluent) were scraped in serum-containing medium. At various times following scraping, nuclei were prepared and run-on analysis was performed as described in Materials and Methods. ^{32}P-labeled nuclear run-on products from equal number of cells at each time point were hybridized to denatured DNA dot-blotted on nitrocellulose filters. The endothelin and actin genes were included as an internal control. The run-on result shown is a representative example of two independent experiments.

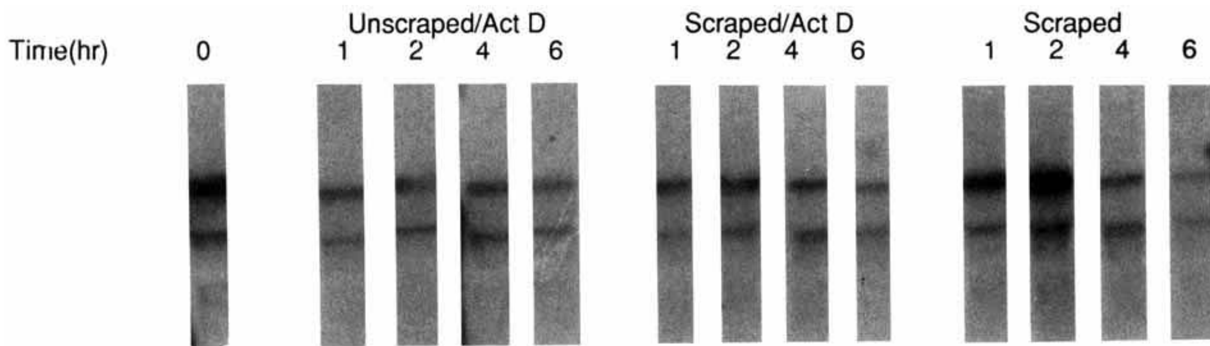


Fig. 10. The effect of scraping on the stability of bFGF mRNA. BAE (80–90% confluent) were treated with 0.5 ng/ml TGF-β1 for 2 h, followed by extensive rinsing to remove the growth factor. The cells were then fed with fresh medium containing actinomycin D to block further transcription. One group of cells received no further treatment whereas a second group was scraped. A third group of cells was scraped without prior actinomycin D treatment. At various times after injury, total RNA was isolated and examined for bFGF mRNA expression by Northern blot analysis.

expected, scraping BAE led to the immediate release of a large proportion of their bFGF. The release of bFGF was followed by a rapid increase in bFGF mRNA and intracellular bFGF levels were restored to starting levels within 6 h of scraping.

Steady state levels of bFGF mRNA in BAE were dramatically increased 2 h following bFGF release. This rapid increase is similar to that observed for c-fos and c-myc following wounding of fibroblasts [Hatanaka and Tsuboi, 1991; Verrier et al., 1986]. Indeed, it has been suggested that the expression of bFGF during the early phase of the response to injury may confer competence for growth [Murphy et al., 1988]. The early increase in bFGF mRNA was followed by a decline to baseline at 6 h after scraping. The rapid reduction in the message is likely to be due to the mRNA instability. The 3′-untranslated region of the bFGF transcripts possess the AUUUA sequence [Abraham et al., 1986] that has been implicated in destabilizing the mRNA of several proto-oncogenes and cytokines [Shaw and Kamen, 1986].

Although we have not investigated the mechanism underlying the increase in bFGF mRNA at 24 h, two observations indicate that this increase is regulated by a different means than the early (at 2 h) increase. First, whereas the inclusion of suramin suppressed the increase at 2 h by up to 90%, it had virtually no effect on the increase observed at 24 h. Second, cycloheximide suppressed more of the increase at 24 h than at 2 h.

Proteases and TGF-β1 in Injury-Induced Increase in bFGF mRNA Level

The versatile role of proteases in regulating various biological processes, such as blood clotting [for review see Stern et al., 1988], processing of propeptides [Ringe, 1992], and embryonic pattern formation [Hecht and Anderson, 1992] is well documented. Our data implicate thrombin and plasmin, two serine proteases, in the regulation of bFGF mRNA. Thrombin, a factor known for its role in the clotting cascade, has

also been shown to be a potent activator of EC, a mitogen for cells of mesodermal origin [Shuman, 1986] and a stimulator of bFGF gene expression in bovine capillary EC [Weich et al., 1991]. The source of thrombin in this model is not known with certainty, but serum does contain thrombin which has shown to be active in other culture systems [Gurwitz and Cunningham, 1988].

Plasmin, a serine protease known for its activity in fibrinolysis, has been implicated in a number of vascular processes including basement membrane degradation [Moscatelli and Rifkin, 1988], release of matrix-bound bFGF [Saksela and Rifkin, 1990], and activation of latent TGF-β1 [Lyons et al., 1988]. EC cultured in serum-containing medium generate plasmin and have surface receptors for serum-derived plasminogen [Hajjar et al., 1986] and for plasminogen activator [Vassalli et al., 1985], which they synthesize. Injury to EC may activate this system by increasing plasminogen activator (see below) and/or by separating plasminogen activator from the matrix-associated PAI-I.

We have also demonstrated a role for TGF-β1 in the increase of bFGF mRNA. EC are known to make TGF-β in a latent form [Antonelli-Orlidge et al., 1989]. Further, it has been shown that exogenous bFGF induces the production of active TGF-β1 via plasmin [Flaumenhaft et al., 1992]. In our model, bFGF released by scraping may increase plasminogen activator, which would mediate the activation of endogenous latent TGF-β1. We have found that relatively low (500 pg/ml) concentrations of TGF-β induce significant (10- to 15-fold) increases in steady state levels of bFGF mRNA (data not shown). Although our data indicate that the direct effect of bFGF on its own gene expression is modest (5 ng/ml induced a 2-fold increase), bFGF may indirectly influence its own expression by activating of TGF-β [Cook et al., 1990; Leof et al., 1986; Lindholm et al., 1990; Madri et al., 1992; Sternfeld et al., 1988; Winkles and Gay, 1991]. It is clear from our studies that bFGF, TGF-β1, plasmin, and thrombin do not account for all of the scrape-induced increases in bFGF mRNA. Although the nature of the additional regulatory activities is unknown, the inhibitory effects of suramin indicate that other soluble factors may play a role. In addition, perturbation resulting from mechanical force might also contribute to the increases in bFGF mRNA.

Molecular Mechanisms Regulating bFGF Gene Expression

The results from the nuclear run-on assay and mRNA stability study indicate that the increase in steady state bFGF mRNA following scraping is due to the transcriptional activation of the bFGF gene. Further, we have found that the increase in bFGF mRNA following scraping is dependent on new protein synthesis, suggesting that scrape injury induces the synthesis of a labile regulatory factor. In the analysis of bFGF mRNA stability, two populations of transcripts with differing stabilities were evident. While a majority of the bFGF mRNA had a half-life of less than 1 h, a sub-population appeared relatively stable throughout the 6 h time course. The mechanism underlying this phenomena is not clear. We have found that bFGF mRNA transcripts are distributed in various subcellular compartments (Ku and D'Amore, unpublished data). The sequestration of the transcripts in some compartments (e.g., in association with the cytoskeleton) may contribute to the relative stability of some of the mRNA.

bFGF as a "Wound Hormone" and Survival Factor

The lack of bFGF secretion, coupled with high cell-associated levels of bFGF in many cell types [Vlodavsky et al., 1987], has led to the speculation that bFGF may function intracellularly as a survival factor. In support of this concept, inclusion of antisense oligonucleotides against bFGF has been reported to lead to a dramatic suppression of EC proliferation [Itoh et al., 1992] and neutralizing antibodies against bFGF reduce the proliferation of EC [D'Amore, 1990; Liaw and Schwartz, 1993]. Suspecting that intracellular and/or paracrine bFGF is important for cell growth and survival, and knowing that sublethal injury causes cultured EC to release bFGF [McNeil, 1991], it seems reasonable that EC might have a feedback system to regulate bFGF expression following sublethal injury. Blocking the restoration of cellular bFGF by the inclusion of suramin or antagonists to thrombin prevented cell proliferation following scrape-induced bFGF release.

Further, sublethal injury of EC appears to be a plausible mechanism for cells to release bFGF. This is especially significant in light of the demonstrated lack of bFGF secretion under normal circumstances [Vlodavsky et al., 1987]. It has been conclusively documented that sublethal in-

jury occurs in vivo. Thus, the known ability of bFGF to act as an endothelial mitogen, a stimulator of connective tissue [Davidson et al., 1988], and an inducer of neural differentiation [Wagner and Kostyk, 1990] may be central to the actions that bFGF plays when it is locally released at wound sites. The apparent need for cells to maintain a constant/steady level of bFGF protein is compatible with the proposed role of bFGF as a "wound hormone" and survival factor.

ACKNOWLEDGMENTS

We gratefully acknowledge Wendy Evanko and Carlene Pavlos for their expertise in editing and preparation of this manuscript; David Shima and Sandra Smith for critical reading of the manuscript; Sandy Dethlefsen for supplying BAE cultures used in this study; and Martin Meyers for performing the experiment described in Figure 1 of this manuscript. This work was supported by NIH grant EY05985 (P.D.).

REFERENCES

Abraham JA, Whang JL, Tumolo A, Mergia A, Friedman J, Gospodarowicz D, Fiddes JC (1986): Human basic fibroblast growth factor: Nucleotide sequence and genomic organization. EMBO J 5:2523–2528.

Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA (1989): An activated form of TGF-β is produced by cocultures of endothelial cells and pericytes. Proc Natl Acad Sci USA 86:4544–4548.

Bost LM, Aotaki-Keen AE, Hjelmeland LM (1992): Coexpression of FGF-5 and bFGF by the retinal pigment epithelium in vitro. Exp Eye Res 55:727–734.

Chirgwin JM, Przbyla AE, MacDonald RJ, Rutter WJ (1979): Isolation of biologically active ribonucleic acid from sources enriched for ribonuclease. Biochemistry 18:5294–3201.

Coffey RJ, Leof EB, Shipley GD, Moses HL (1987): Suramin inhibition of growth factor receptor binding and mitogenicity in AKR-2B cells. J Cell Physiol 132:143–148.

Cook PW, Coffey RJ, Magun BE, Pittelkow MR, Shipley GD (1990): Expression and regulation of mRNA coding for acidic and basic fibroblast growth factor and transforming growth factor α in cells derived from human skin. Mol Endocrinol 4:1377–1385.

D'Amore PA (1990): Modes of FGF release in vivo and in vitro. Cancer Metastasis Rev 9:227–238.

Davidson J, Buckley A, Woodward S, Nichols W, McGee G, Demetriou A (1988): Mechanisms of accelerated wound repair using epidermal growth factor and basic fibroblast growth factor. Prog Clin Biol Res 266:63–75.

Davies PF (1988): Endothelial cells, hemodynamic forces, and the localization of atherosclerosis. In Ryan VS (ed): "Endothelial Cells." Boca Raton, FL: CRC Press, pp 123–138.

Finklestein SP, Apostolides PJ, Caday CG, Prosser J, Philips MF, Klagsbrun M (1988): Increased basic fibroblast growth factor (bFGF) immunoreactivity at the site of focal brain wounds. Brain Res 460:253–259.

Flaumenhaft R, Abe M, Mignatti P, Rifkin DB (1992): Basic fibroblast growth factor-induced activation of latent transforming growth factor β in endothelial cells: Regulation of plasminogen activator activity. J Cell Biol 118:901–909.

Florkiewicz RZ, Shibata F, Barankiewicz T, Baird A, Gonzalez A-M, Florkiewicz E, Shah N (1991): Basic fibroblast growth factor gene expression. Ann NY Acad Sci 638:109–126.

Fort P, Marty L, Piechaczyk M, El Sabrouty S, Dani C, Jeanteur P, Blanchard JM (1985): Various rat adult tissues express only one major mRNA species from the glyceradehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res 13:1431–1442.

Gajdusek CM, Carbon S (1989): Injury-induced release of basic fibroblast growth factor from bovine aortic endothelium. J Cell Physiol 139:570–579.

Gay CG, Winkles JA (1991): Interleukin 1 regulates heparin-binding growth factor 2 gene expression in vascular smooth muscle cells. Proc Natl Acad Sci USA 88:296–300.

Goldsmith KT, Gammon RB, Garver RI (1991): Modulation of bFGF in lung fibroblasts by TGF-β and PDGF. Am J Physiol 261:L378–L385.

Gospodarowicz D, Moran J, Braun D, Birdwell CR (1976): Clonal growth of bovine endothelial cells in culture: Fibroblast growth factor as a survival factor. Proc Natl Acad Sci USA 73:4120–4124.

Gurwitz D, Cunningham DD (1988): Thrombin modulates and reverses neuroblastoma neurite outgrowth. Proc Natl Acad Sci USA 85:3440–3444.

Hajjar KA, Harpel PC, Jaffe EA, Nachman RL (1986): Binding of plasminogen to cultured human endothelial cells. J Biol Chem 261:11656–11662.

Hatanaka M, Tsuboi K (1991): Initiation of wound healing by proteinases released from damaged cells. Int J Tissue React XIII:249–255.

Hecht PM, Anderson KV (1992): Extracellular proteases and embryonic pattern formation. Trends Cell Biol 2:197–202.

Hosang M (1985): Suramin binds to platelet-derived growth factor and inhibits its biological activity. J Cell Biochem 29:265–273.

Ii M, Yoshida H, Aramaki Y, Masuya H, Hada T, Terada M, Hatanaka M, Ichimori Y (1993): Improved enzyme immunoassay for human basic fibroblast growth factor using a new enhanced chemiluminescence system. Biochem Biophys Res Commun 193:540–545.

Itoh H, Mukoyama M, Pratt R, Dzau VJ (1992): Specific blockade of basic fibroblast growth factor gene expression in endothelial cells by antisense oligonucleotide. Biochem Biophys Res Commun 188:1205–1213.

Kavanaugh WM, Harsh GR, Starksen NF, Rocco CM, Williams LT (1988): Transcriptional regulation of the A and B chain genes of platelet-derived growth factor in microvascular endothelial cells. J Biol Chem 263:8470–8472.

Klagsbrun M, D'Amore PA (1991): Regulators of angiogenesis. Ann Rev Physiol 53:217–239.

Klagsbrun M, Vlodavsky I (1988): Biosynthesis and storage of basic fibroblast growth factor (bFGF) by endothelial cells: Implication for the mechanism of action of angiogenesis. Prog Clin Biol Res 266:55–61.

Kostyk SK, D'Amore PA, Herman IM, Wagner JA (1994): Optic nerve injury alters basic fibroblast growth factor localization in the retina and optic tract. J Neurosci 14:1441–1449.

Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL (1986): Induction of c-sis mRNA and activity similar to platelet derived growth factor by transforming growth factor-β: A proposed model for indirect mitogenesis involving autocrine activity. Proc Natl Acad Sci USA 83:2453–2457.

Liaw L, Schwartz SM (1993): Comparison of gene expression in bovine aortic endothelium in vivo versus in vitro. Arteriosclerosis Thrombosis 13:985–993.

Lindholm D, Hengeren B, Zafra F, Thoenen H (1990): Transforming growth factor-β1 stimulates expression of nerve growth factor in the rat CNS. Neuro Rep 1:9–12.

Lyons RM, Keski-Oja J, Moses H (1988): Proteolytic activation of latent transforming growth factor-β from fibroblast conditioned medium. J Cell Biol 106:1659–1665.

Madri JA, Bell L, Merwin JR (1992): Modulation of vascular cell behavior by transforming growth factor-β. Mol Reprod Dev 32:121–126.

McNeil PL (1991): Cell wounding and healing: The opening and closing of wounds in the cell membrane provides a route for the release of molecules that maintain and repair tissue. Am Sci 79:222–235.

McNeil PL, Ito S (1989): Gastrointestinal cell plasma membrane wounding and resealing in vivo. Gastroenterology 96:1238–1248.

McNeil PL, Muthukrishnan L, Warder E, D'Amore PA (1989): Growth factors are released by mechanically wounded endothelial cells. J Cell Biol 109:811–822.

Moscatelli D, Rifkin DB (1988): Membrane and matrix localization of proteinases: A common theme in tumor cell invasion and angiogenesis. Biochim Biophys Acta 948:67–85.

Moscatelli D, Presta M, Rifkin DB (1986): Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis and migration. Proc Natl Acad Sci USA 83:2091–2095.

Murphy PR, Sata R, Sato Y, Friesen HG (1988): Fibroblast growth factor messenger ribonucleic acid expression in a human astrocytoma cell line: Regulation by serum and cell density. Mol Endocrinol 2:591–598.

Muthukrihnan L, Warder E, McNeil P (1991): Basic fibroblast growth factor is efficiently released from a cytosolic storage site through plasma membrane disruptions of endothelial cells. J Cell Physiol 148:1–16.

Padua RR, Kardami E (1993): Increased basic fibroblast growth factor (bFGF) accumulation and distinct patterns of localization in isoproterenol-induced cardiomyocyte injury. Growth Factors 8:291–306.

Pollack M, Richard M (1990): Suramin blockade of insulin-like growth factor 1-stimulated proliferation of human osteosarcoma cells. J Natl Cancer Inst 82:1349–1352.

Ringe D (1992): Proteases: To each his own. Nature 356:748–749.

Roberts AB, Sporn MB (1990): The transforming growth factor-βs. In Sporn MB, Roberts AB (eds): "Handbook of Experimental Pharmacology: Peptide Growth Factors and Their Receptors." Berlin: Springer-Verlag, pp 419–472.

Saksela O, Rifkin DB (1990): Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. J Cell Biol 110261:767–776.

Sato Y, Rifkin DB (1988): Autocrine activities of basic fibroblast growth factor: Regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. J Cell Biol 107:1199–1205.

Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D (1987): Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 325:257–259.

Shaw G, Kamen R (1986): A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46:659–667.

Shuman MA (1986): Thrombin-cellular interactions. Ann NY Acad Sci 485:349–368.

Stern DM, Handley D, Nawroth PP (1988): Endothelial cell regulation of coagulation. In Clark RAF, Henson PM (eds): "The Molecular and Cellular Biology of Wound Repair." New York: Plenum Press, pp 87–114.

Sternfeld MD, Hendrickson JE, Keeble WW, Rosenbaum JT, Robertson JE, Pittelkow MR, Shipley GD (1988): Differential expression of mRNA coding for heparin-binding growth factor type 2 in human cells. J Cell Physiol 136:297–304.

Thomas KA (1988): Transforming potential of fibroblast growth factor genes. Trends Biochem Sci 13:327–328.

Vassalli J-D, Baccino D, Belin D (1985): A cellular binding site for the M_r 55,000 form of the human plasminogen activator, urokinase. J Cell Biol 100:86–92.

Verrier B, Müller D, Bravo R, Müller R (1986): Wounding a fibroblast monolayer results in the rapid induction of the c-fos protooncogene. EMBO J 5:913–917.

Vlodavsky I, Fridman R, Sullivan R, Sasse J, Klagsbrun M (1987): Aortic endothelial cells synthesize basic fibroblast growth factor which remains cell associated and platelet-derived growth factor-like protein which is secreted. J Cell Physiol 131:402–408.

Wagner JA, Kostyk SK (1990): Regulation of neural cell survival and differentiation by peptide growth factors. Curr Opin Cell Biol 2:1050–1057.

Weich HA, Iberg N, Klagsbrun M, Folkman J (1991): Transcriptional regulation of basic fibroblast growth factor gene expression in capillary endothelial cells. J Cell Biochem 47:158–164.

Winkles JA, Gay CG (1991): Serum, phorbol ester, and polypeptide mitogens increase class 1 and 2 heparin-binding (acidic and basic fibroblast) growth factor gene expression in human vascular smooth muscle cells. Cell Growth Differ 2:531–540.

Witte L, Fuks Z, Haimovitz FA, Vlodavsky I, Goodman DS, Eldor A (1989): Effects of irradiation on the release of growth factors from cultured bovine, porcine, and human endothelial cells. Cancer Res 49:5066–5072.

Wright HP (1968): Endothelial mitosis around aortic branches in normal guinea pigs. Nature 220:78–79.