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HUMAN DERMAL FIBROBLASTS EXPRESS MULTIPLE bFGF AND aFGF PROTEINS

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SUMMARY

We investigated the regulation of expression of bFGF and aFGF in cultures of normal human dermal fibroblasts grown in a defined, serum-free medium which did not contain FGF. Under these conditions we detected three molecular weight forms of bFGF protein [18.0, 23.0, and 26.6 kiloDaltons (kD)] and three molecular weight forms of aFGF protein (18.4, 19.2, and 28.6 kD) in these cells using western blot analysis. The addition of fetal bovine serum (FBS) to these cultures caused an accumulation of all three molecular weight forms of bFGF protein with a more dramatic accumulation of the 23.0 and 26.6 kD forms. In contrast, the addition of FBS to the cultures had no effect on the level of aFGF proteins. Analysis of mRNA isolated from cells grown in serum-free medium revealed multiple species of both bFGF and aFGF RNA with molecular weights that correlated with our previous observations. The abundance of all bFGF mRNA species increased dramatically after serum treatment while the abundance of aFGF mRNA species increased only slightly. Our observations demonstrate that factor(s) present in FBS elevate the levels of bFGF mRNA and protein beyond the levels already present in the cultures growing in serum-free medium. Moreover, both bFGF and aFGF protein are present in these cells as multiple molecular weight species. Some of these forms are higher in apparent molecular weight than would be predicted from ATG-initiated primary translation products of these genes. We also show that the cells used for this study proliferate in response to bFGF and aFGF, thus, it is possible that the growth of these cells could be subject to autocrine/paracrine control in certain conditions.

Key words: bFGF; aFGF; human dermal fibroblasts; serum-free medium.

INTRODUCTION

Basic and acidic fibroblast growth factors (bFGF and aFGF) are members of the heparin binding growth factor family. Currently, there are seven members of this family: bFGF, aFGF, int-2, hst/K-FGF, FGF-5, FGF-6, and KGF (2,12,17). These peptides have been shown to have a broad spectrum of biological effects on the growth and differentiation of a variety of mesodermally and neuroectodermally-derived cells in culture (2,28).

Cultured human fibroblasts have been shown to express multiple mRNA species for both bFGF (26) and aFGF (16) as well as active bFGF protein (27). In addition, normal human dermal fibroblasts have been shown to proliferate in response to bFGF and aFGF (24). Analysis of bFGF and aFGF cDNAs revealed that the AUG-initiated, primary translation product of both of these growth factors is 155 amino acids in length (1,16). Recently, molecular weight forms of bFGF protein higher than that predicted from initial sequence analysis of the cDNA have been identified (7,20). Experiments using human hepatoma cell-derived cDNAs in both in vitro translation assays and COS-1 cell transfectants revealed multiple species of bFGF protein. From analysis of these experiments, Prats et al. identified three sizes of bFGF protein (18, 21, and 22.5 kD) and Florkiewicz et al. identified four sizes of bFGF protein (17, 22.5, 23.1, and 24.2 kD). Evidence from site-directed mutagenesis presented in these studies suggests these higher molecular weight forms of bFGF arise from non-AUG start codons 5′ to the predicted AUG start codon (*see* Figure 1, below).

It has been hypothesized that altered responsiveness to or altered production of growth factors by cells may occur during wound healing or result in pathological states such as cancer (8). Our previous results (16,26) demonstrating that aFGF and bFGF mRNA are produced by human dermal fibroblasts prompted us to determine whether both bFGF and aFGF proteins are produced by these cells. In the current study, we show that both growth factors are produced in multiple, "higher" molecular weight forms and that the production of bFGF proteins can be regulated by serum treatment of these cells.

MATERIALS AND METHODS

Reagents. Medium MCDB 202a was prepared in our laboratory according to previously published procedures (9). Human recombinant EGF was obtained from Amgen (Thousand Oaks, CA). Bovine serum albumin (BSA) was obtained from ICN (Cleveland, OH, cat #810013). Insulin (cat #I-5500), trypsin (cat #T-8253), heparin acrylamide beads, molecular weight markers for protein gels, and all other chemicals unless noted, were obtained from Sigma Chemical Co. (St. Louis, MO). Calf serum was obtained from HyClone Laboratories (Logan, UT). Fetal bovine serum (FBS) was

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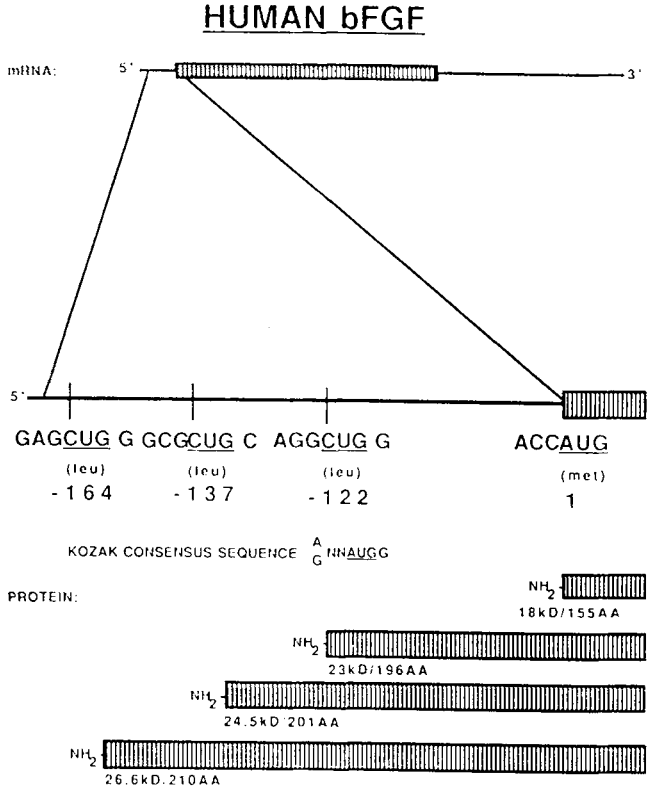

FIG. 1. Schematic drawing of human bFGF mRNA and protein. Potential CUG initiation codons are shown. Numbers refer to the position in bp from AUG start site. The three CUG codons upstream from the AUG start site are shown with their surrounding base sequences. CUG codons located at positions −122 and −164 contain the Kozak consensus sequence (*see* text). The peptides which could arise from the AUG and CUG translational start sites are diagrammed. The number of amino acids which would be contained in the primary translation products and the calculated molecular mass in kilodaltons (kD) of the various forms are indicated.

obtained from GIBCO (Grand Island, NY). Tissue culture plasticware was obtained from Corning.

Cell culture. Human neonatal dermal foreskin fibroblasts (strain NFF-5) were isolated from normal tissue by collagenase digestion as previously described (26). Human adult dermal fibroblasts were obtained from ATCC (CRL1505). Stock cultures of fibroblasts were maintained in medium MCDB 202a supplemented with 5% (v/v) calf serum. Cultures used for experiments were between population doubling level 8 and 25. Quiescent stock cultures were trypsinized using ice cold 0.05% trypsin and resuspended in medium MCDB 202a supplemented with 5% calf serum to neutralize the trypsin. The cells were recovered by centrifugation at 180 $\times g$ and the cell pellet was resuspended in medium MCDB 202a supplemented with 5 ng/ml EGF, 1 μg/ml insulin, and 100 μg/ml BSA. Cells were then plated in 10 cm plates at 2.5×10^3 cells/cm^2 and grown for 7 days without a medium change. On day 7, the medium was changed to fresh medium as above, and the cultures were incubated for an additional 3 days. On day 10 when the cultures had formed a complete monolayer on the surface of the dish without multilayering, FBS was added directly to the medium and the cells were allowed to incubate for 8–24 hours before collection of the samples as described in the text and legends to figures.

Human neonatal keratinocytes (NHEK 239) were obtained from Clonetics Corporation (San Diego, CA) and stock cultures grown in complete medium (supplemented medium KBM, Clonetics) as previously described (4,24). For isolation of mRNA from keratinocyte cultures, secondary cultures of keratinocytes were trypsinized from stock culture flasks and plated in complete medium at a density of 5×10^3 cells/cm^2 in 10 cm culture dishes as previously described. After 3 days of incubation, the plates were washed with buffered saline solution A (10.0 m*M* glucose; 3.0 m*M* KCl; 130.0 m*M* NaCl; 1.0 m*M* $Na_2HPO_4 \cdot 7H_2O$; 0.0033 m*M* phenol red; 30.0 m*M* HEPES) and the medium replaced with medium KBM supplemented with 5×10^{-7} *M* hydrocortisone (standard medium) as previously described. After 2 days of growth in standard medium, the medium was changed to fresh standard medium, standard medium supplemented with 4.5 mg/ml mAb Lal (an EGF receptor antagonist; Upstate Biotechnology, Inc., Lake Placid, NY) or 10 ng/ml transforming growth factor type-α (a gift from Dr. Rik Derynck, Genentech, Inc.). 24 Hours later the medium was removed and poly A+ RNA isolated from the cells as described. These treatments which regulate the level of TGF-α mRNA in keratinocytes were used as negative controls for bFGF hybridization.

Preparation of cell lysates and western blot analysis. Briefly, cells were lysed in a buffer containing 1% NP-40 and removed from the culture dish. Nuclei were removed by centrifugation and the lysates were incubated with heparin-acrylamide beads. Bound material was eluted from the beads by the addition of sodium dodecyl sulfate (SDS) containing polyacrylamide gel electrophoresis sample buffer. The equivalent of two 10 cm plates was loaded onto each lane of the gel. A single 10-cm plate of cells corresponds to approximately 3.6×10^6 cells and 4.36×10^6 cells for serum-free and 24-hour serum treated cultures, respectively. The samples were electrophoresed on a 12% SDS polyacrylamide gel and transferred, electrophoretically, to nitrocellulose membranes. The blots were subsequently incubated with 1:6000 dilution of an ascites fluid containing a mouse monoclonal antibody (148.6.1.1) raised against human.recombinant bFGF (a gift from Dr. C. Hart, Zymogenetics, Inc., Seattle, WA) or a 1:1000 dilution of a rabbit polyclonal antibody (A2) raised against human recombinant aFGF (a gift from Dr. Ken Thomas, Merck Institute, Rathway, NJ). bFGF polyclonal antibody raised against a synthetic decapeptide representing amino acids 24–33 of human bFGF was the gift of Dr. E. Smith, Children's Hospital Medical Center, Cincinnati, OH. Following primary antibody incubation, the membranes were incubated with a 1:7500 dilution of an alkaline phosphatase conjugated antimouse IgG (Promega, Madison, WI) and developed in BCIP/NBT substrates.

Specificity of the monoclonal antibody (mAb) was determined by two methods. First, incubation of western blots containing both purified aFGF (10–100 ng/lane) and bFGF (10 ng/lane) with the bFGF mAb followed by processing as described demonstrated that the mAb reacted only with bFGF. Second, we crosslinked human recombinant bFGF to an affinity support column (Affigel 10 Biorad) that immobilizes the protein via crosslinking to its primary amino group. Ascites fluid containing the bFGF mAb was passed over the column so as to remove bFGF-specific immunoglobins from the fluid. Analysis of the ascites fluid that passed through the column via western blot showed no immunoreactive bands (data not shown). Treatment of the column at high pH and subsequent use of the eluant on western blots containing fibroblast lysates re-

vealed three molecular forms on bFGF protein. Specificity of the aFGF antibody is demonstrated in the text and figures.

Poly-A+ mRNA isolation. Preparation of RNA for Northern blot analysis was performed as previously described (26). Poly A+ mRNA was purified by oligo(dT)-cellulose chromatography.

Probes. A cRNA bFGF probe corresponding to the first 530 nucleotides of sequence 3′ to the translational stop codon were synthesized from plasmid pGb530 which consists of a 530 bp BalI/EcoRI restriction fragment of human bFGF cDNA cloned into the Sma I site of the vector pGEM4Z (Promega). cRNA probes corresponding to the cyclophilin gene (1B15) were synthesized from a 680 bp cDNA insert in Sp65-1B15 (6). cRNA probes for aFGF corresponding to the first 479 nucleotides of the protein coding sequence were synthesized from plasmid pJC3-5 which consists of a 479 NcoI/EcoRI restriction fragment of human aFGF cDNA cloned into the Sma I site of vector pGEM4Z (Promega) (16). cRNA reactions were carried out according to the method of Melton et al. (15).

Northern blots. Northern blots were prepared as previously described (26). RNA size markers were obtained from Bethesda Research Laboratories. Hybridization of probes was conducted for 18 hours at 60° C using $1–5 \times 10^6$ cpm/ml of probe in hybridization mix as described previously (26). Autoradiography was performed using Kodak X-Omat film with an intensifying screen at −80° C for 6–24 hours.

DNA synthesis assays. For measurement of ^{3}H-thymidine incorporation into DNA, NFF-5 cells were removed from stock culture flasks, resuspended and centrifuged as described above. The cells were then resuspended in cold medium MCDB 202a supplemented with 5.0 μg/ml insulin and plated at 1×10^4 cells/cm^2 in 24 well culture dishes. The cells were incubated for 72 hours without a medium change. After the first 48 hours of the incubation, various concentrations of growth factors with or without heparin sulfate were added to some wells and the incubation continued. After 68 hours of incubation, 1.0 μCi/ml ^{3}H-thymidine was added to each well in a small volume. At the end of the incubation, the medium was removed and the cells were fixed with cold 10% TCA. Relative incorporation of ^{3}H-thymidine into TCA insoluble material was determined as previously described (25). Recombinant bFGF and aFGF were the gifts of Drs. J. Abraham (California Biotechnology) and K. Thomas (Merck), respectively.

Results

Both bFGF and aFGF mRNAs are expressed in dermal fibroblasts grown in serum-free medium. Previous studies have demonstrated the presence of multiple species of bFGF and aFGF mRNA in human neonatal dermal fibroblasts grown in serum-free medium containing bFGF, or in medium containing FBS (16,26). To facilitate the examination of FGF proteins, we decided to grow human neonatal dermal fibroblasts in growth medium lacking serum and FGF. Fibroblasts were harvested from stock culture flasks, plated and grown in medium MCDB 202a supplemented with EGF, insulin and BSA as described in the Materials and Methods. When the cultures reached high density (*see* Materials and Methods), we harvested RNA from serum-free or acutely serum-treated cultures and examined the expression of aFGF and bFGF mRNA by Northern blot analysis (Fig. 2). Because mRNA collected from normal human keratinocytes has been shown to express very little bFGF and aFGF mRNA, it was used as a negative control on our Northern blots (26).

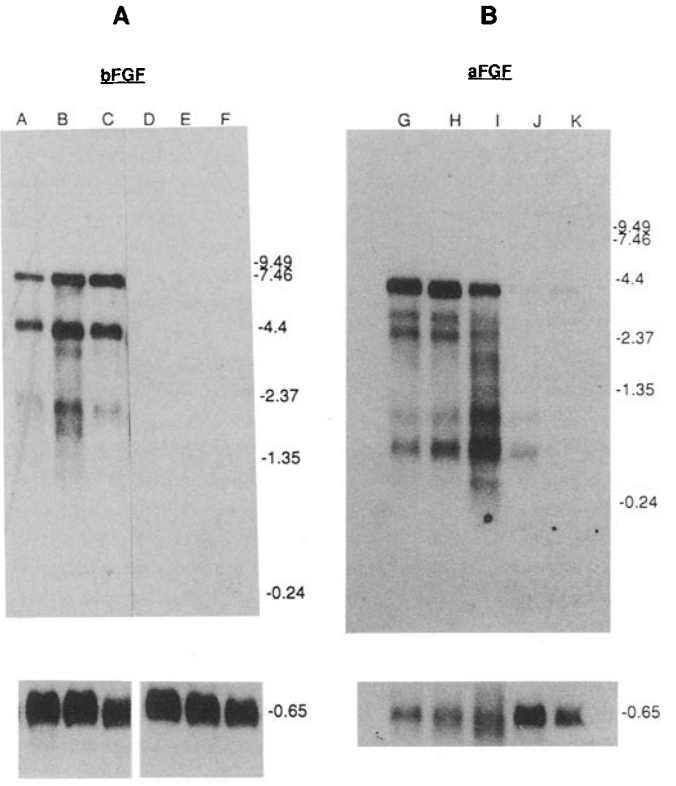

FIG. 2. Northern blot analysis of poly-A+ RNA from normal human dermal fibroblasts and human keratinocytes. 3.0 μg of poly-A+ RNA from normal human dermal fibroblasts or 5 μg of poly-A+ RNA from normal human keratinocytes was loaded into each lane. *Panel A.* Northern blot hybridized with a probe specific for bFGF. *Lane A*, fibroblasts grown in serum-free medium (control); *lane B*, fibroblasts grown in serum-free medium + 5% FBS for 4 hours; *lane C*, fibroblasts grown in serum-free medium + 5% FBS for 24 hours; *lane D*, keratinocytes grown in serum-free medium; *lane E*, keratinocytes grown in serum-free medium + TGFα (10 ng); *lane F*, keratinocytes grown in serum-free medium + anti-EGF receptor antibody (10 n*M*). *Panel B*: Northern blot hybridized with a probe specific for aFGF. *Lane G*, fibroblasts grown in serum-free medium (control); *lane H*, fibroblasts grown in serum-free medium + 5% FBS for 4 hours; *lane I*, fibroblasts grown in serum-free medium + 5% FBS for 24 hours; *lane J*, keratinocytes grown in serum-free medium + TGFα (10 ng); *lane K*, keratinocytes grown in serum-free medium + anti-EGF receptor antibody (10 n*M*). A probe specific for the constitutive gene, cyclophilin (1B15), was hybridized to both blots and appears as the 0.65 kb band (shown at the bottom). Migration of RNA ladder (kb $\times 10^{-3}$) is on the right.

As a control for variation in loading the gels and capillary transfer we subsequently probed the same blot with cRNA encoding a constitutively expressed gene, cyclophilin (1B15) (Fig. 2., *bottom panel*). As shown in Figure 2; *panel A*, fibroblasts express three predominant bFGF mRNA species with molecular sizes of 7.1, 4.1, and 1.8 kilobases (*lanes B* and *C*). Two additional bFGF mRNA species were evident in the serum-stimulated cultures with sizes of 3.2 and 1.4 kilobases. As previously reported (26), the relative abundance of the bFGF mRNAs increased with serum treatment. No bFGF mRNA transcripts were detected in RNA from human keratinocytes grown in serum-free medium (Figure 2; *panel A, lanes D–F*) as previously reported (26). A similar blot was probed with an aFGF cRNA to determine if aFGF mRNA could be detected in fibroblasts grown under the same conditions. As shown in Figure 2;

panel B, multiple species of aFGF mRNA with sizes of 3.9, 2.8, 2.2, 0.8, and 0.5 kb were detected in these cells. The abundance of all aFGF mRNA species increased slightly after 4 hours of FBS treatment. The level of the major aFGF transcript at 3.9 kb decreased after 24 hours of serum treatment, however, levels of aFGF mRNA transcripts at 0.8 and 0.5 kb increased. The sizes of the aFGF and bFGF mRNA species detected under these conditions are similar if not identical to those previously reported by us and others (16,26). Thus, we have shown that fibroblasts grown continuously in serum-free medium lacking FGF produce multiple species of both aFGF and bFGF mRNA, and that under these conditions bFGF mRNA abundance can be elevated by serum treatment.

Fibroblasts contain multiple bFGF proteins that increase in abundance with serum treatment. To test whether bFGF protein was present in fibroblasts and regulated by serum exposure, we analyzed bFGF proteins in these cells by western blot analysis using a monoclonal anti-bFGF antibody. Cultures were grown to high density in serum-free medium and then treated with 5% (v/v) FBS as in the experiments described above. Cell lysates were collected after 8 hours of serum treatment and incubated with heparin-acrylamide beads. Bound material was eluted with SDS-PAGE sample buffer, electrophoresed on SDS-polyacrylamide gels, and western blot analysis performed as described in Materials and Methods. Human recombinant bFGF protein (154 amino acid form) was used as a standard. As demonstrated in Fig. 3, the monoclonal antibody reacted with three proteins in the fibroblast lysates with apparent molecular weights of 26.6, 23 and 18 kD. An accumulation of all three molecular forms of bFGF was seen in serum-treated cultures compared to the controls (serum-free cultures). A more dramatic accumulation of the 26.6 and 23 kD forms was evident.

A rabbit anti-bFGF polyclonal antibody raised against a synthetic decapeptide representing amino acids 24–33 of human bFGF was also used in the analysis of our western blots. This antisera also detected 18.0, 23.0, and 26.6 kD proteins in fibroblast lysates. Preincubation of this polyclonal antibody with the decapeptide prior to exposure to blots containing lysates blocked the ability of the antibody to recognize any of the forms of bFGF (data not shown). The fact that a different anti-bFGF antibody recognizes the same proteins confirms that the proteins detected are, indeed, bFGF. Thus, we have shown that fibroblasts produce three molecular forms of bFGF protein and that serum treatment of these cultures causes an accumulation of all three forms.

Increases in general protein synthesis with FBS treatment of the culture does not explain the accumulation of bFGF proteins. When cell lysates from identical cultures were analyzed by western blot with either the anti-bFGF monoclonal antibody (mAb) or an anti-aFGF polyclonal antibody, an accumulation of three species of bFGF protein was apparent, however, no accumulation in aFGF protein was seen (*see*, for example, Figure 4). We have also analyzed FBS for the presence of bFGF protein. FBS was incubated with heparin-acrylamide beads and the bound material was eluted with SDS-PAGE sample buffer, electrophoresed on SDS-polyacrylamide gels, and western blot analysis performed using the anti-bFGF mAb. Under these conditions no immunologically reactive bFGF bands were detected.

To determine if the production of multiple forms of bFGF protein was a phenomenon of this particular strain of fibroblasts or the age of the donor, we cultured human adult dermal fibroblasts in the same fashion and obtained cell lysates. Adult fibroblast cell lysates were treated identically to cell lysates from human neonatal fibroblasts. All three forms of bFGF protein, molecular weights of 26.6, 23, and 18 kD, were present in adult fibroblasts, and an accumulation of the three molecular forms was seen in the serum-treated cultures (data not shown).

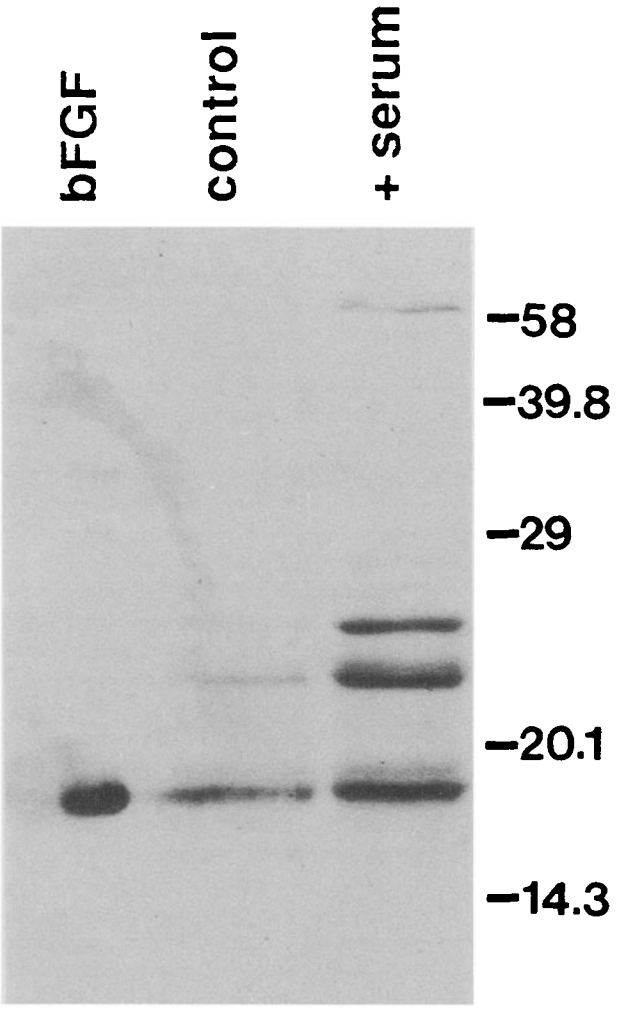

FIG. 3. Western blot analysis of neonatal normal human dermal fibroblast cell lysate. Normal human dermal fibroblasts were grown in serum-free medium (control) or treated for 8 hours with 5% (v/v) FBS (+serum). Cell lysates were collected and western blot analysis performed as described in Materials and Methods. Cell lysates from 2 plates (*see* Materials and Methods) were electrophoresed in each lane. 10 ng of human recombinant bFGF (154 AA form) was loaded in the *left lane*. Migration of molecular weight markers (MW $\times 10^{-3}$) is indicated on the *right side*.

We determined the effect of varying doses of FBS on our ability to detect multiple bFGF proteins. Neonatal fibroblasts were grown in serum-free medium as above and then treated with 0.1, 1.0, 2.5, 5.0, and 10.0 percent (v/v) FBS for 8 hours. Western blot analysis was performed to determine the relative abundance of bFGF proteins. As shown in Figure 5, an accumulation of all three molecular forms of bFGF (18, 23, and 26.6 kD) was seen with serum treatment. The 26.6 and 23 kD forms of bFGF appear to increase more dramatically with serum-treatment compared to the 18 kD form.

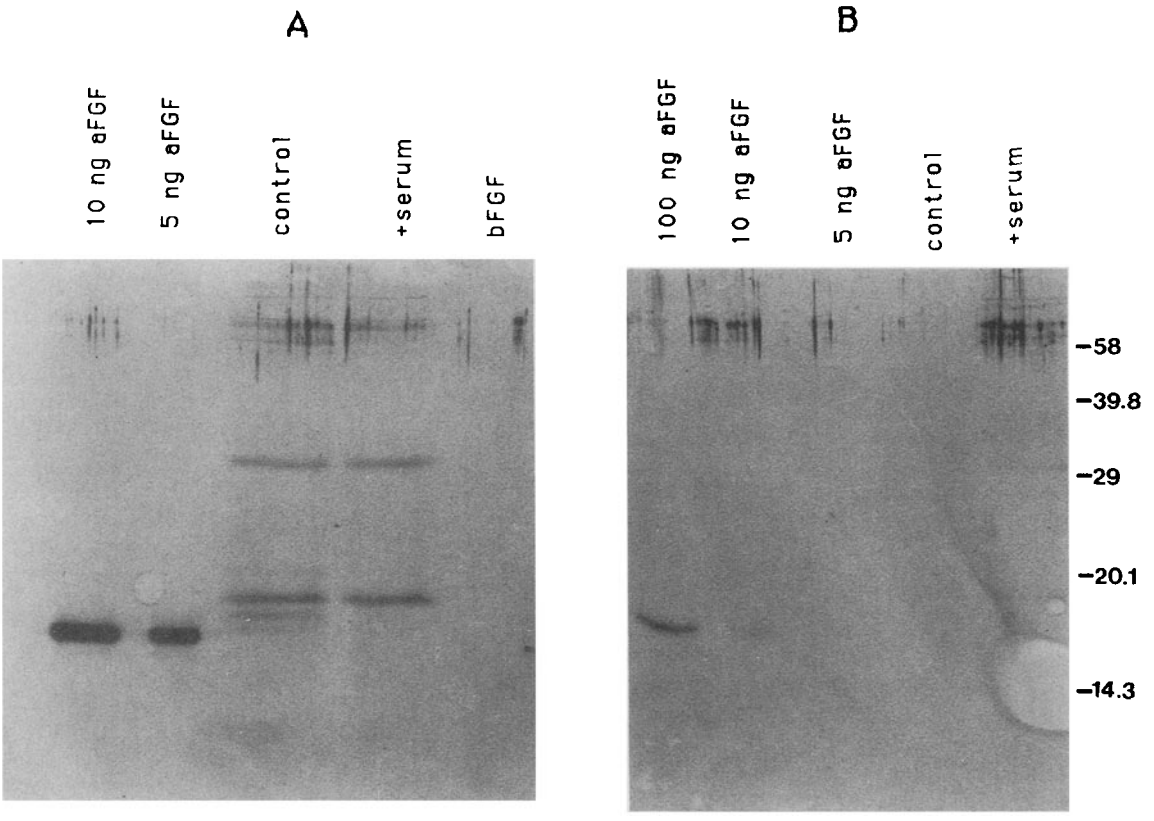

FIG. 4. Western blot analysis of normal human dermal fibroblast cell lysate using an anti-aFGF antibody. Cells were grown as described in Figure 3. Cell lysates from 2 plates (*see* Materials and Methods) were electrophoresed in each lane. *Panel A*: Blot treated with the rabbit polyclonal anti-aFGF antibody. 10 and 5 ng of purified human recombinant aFGF (141 amino acid form) was loaded as a positive control. 10 ng of purified human recombinant bFGF (154 AA form) was loaded as a negative control. *Panel B*: Blot treated with the rabbit polyclonal anti-aFGF antibody preincubated with excess purified human recombinant aFGF (*see* Results). 100, 10 and 5 ng purified human recombinant aFGF (141 AA form) were loaded as controls. Migration of molecular weight markers (MW $\times 10^{-3}$) is indicated on the right side.

Treatment of cultures with a serum concentration of 2.5% FBS or greater appeared to cause a maximal accumulation of bFGF proteins. In this experiment a 40 kD immunoreactive protein was detected in all cultures, but did not increase with serum-treatment. Multiple washings of the heparin-acrylamide beads did not reduce the immunoreactivity of this 40 kD protein migrating in the region of the 39.8 molecular weight marker. This immunoreactive band was not detected in all experiments. In addition, the 40 kD protein does not appear to be an artifact of serum treatment as it is present with the same relative intensity in untreated (control) cultures. We do not know what the 40 kD protein represents at this time. An additional immunoreactive band is seen migrating with the 58 kD marker in Figures 2 and 3. This band appears to be the result of serum treatment of the cultures. It is likely that this 58 kD band is caused by nonspecific interactions of the protein with the heparin-acrylamide beads as multiple washings of the heparin-acrylamide beads with 0.15 *M* NaCl solution prior to elution with sample buffer dramatically decreases the intensity of this band.

Multiple aFGF immunoreactive species are present in fibroblast lysates. We questioned whether the expression of other members of the FGF family could also be regulated by serum. It has previously been shown that fibroblasts produce multiple species of aFGF mRNA (16,29). To test whether fibroblasts produce aFGF protein and if aFGF protein expression could be regulated by serum, neonatal fibroblast cultures were grown to confluency in serum-free medium and stimulated with 5% (v/v) FBS as described above. Cell lysates were collected and western blot analysis was performed as described except that a rabbit polyclonal antibody raised against aFGF was used for detection of immunoreactive species. In fibroblast cell lysates, we detected two prominent immunoreactive bands with molecular weights of 28.6, 19.2 kD (Figure 4, *panel A*). An additional species of 18.4 kD (Figure 4, *panel A*) was also occasionally detected. No accumulation of these three species was seen in the serum-treated cultures compared to the control cultures. A 141 amino acid form of human recombinant aFGF was used as a standard and migrated with a molecular weight 16.6 kD. The anitbody did not detect purified human recombinant bFGF (Fig. 4, *panel A*), and preincubating the antibody with excess purified human recombinant aFGF prior to incubating the antibody with the blot (Figure 4, *panel B*) blocked the detection of all of these bands except the highest level of purified aFGF tested (100 ng/lane). Thus, fibroblasts appear to produce three molecular forms of aFGF protein which (like the mRNA for this growth factor) do not appear to increase dramatically after serum treatment.

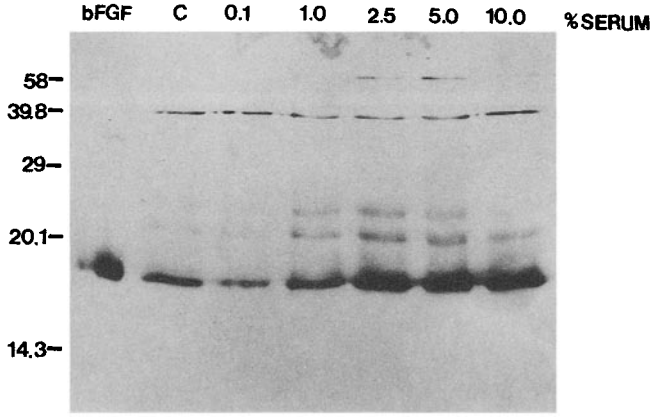

FIG. 5. Western blot analysis of normal human dermal fibroblast cell lysate from cultures treated with varying concentrations of FBS. Cells were grown in serum-free medium and treated with the indicated concentration of FBS for 8 hours as described in Materials and Methods. Cell lysates from 2 plates (*see* Materials and Methods) were electrophoresed in each lane. bFGF: 10 ng human recombinant bFGF (154 AA form). C: Cell lysate from cultures grown in serum-free medium. 0.1: Cell lysate from cultures grown in serum-free medium treated with 0.1% (v/v) FBS for 8 hours. 1.0: cultures grown in serum-free medium treated with 1.0% (v/v) FBS for 8 hours. 2.5: Cell lysate from cultures grown in serum-free medium treated with 2.5% (v/v) FBS for 8 hours. 5.0: Cell lysate from cultures grown in serum-free medium treated with 5.0% (v/v) for 8 hours. 10: Cell lysate from cultures grown in serum-free medium treated with 10% (v/v) FBS for 8 hours. Migration of molecular weight markers (MW $\times 10^{-3}$) is indicated on the *left side*.

Purified aFGF and bFGF stimulate DNA synthesis in human fibroblasts in serum-free medium. We used the purified recombinant aFGF and bFGF which served as controls in our western blot experiments to examine the mitogenicity of these growth factors on the neonatal fibroblasts used for our study. We tested both of the factors over a broad range of concentrations in the presence and absence of heparin sulfate in a serum-free thymidine incorporation assay (*see* Materials and Methods). As shown in Figure 6, these mitogens were nearly equipotent in stimulating DNA synthesis in these cells in the presence of heparin sulfate (bFGF ED_{50}=10 pg/ml; aFGF ED_{50}=40 pg/ml). However, when added to the cultures in the absence of heparin, aFGF was much less effective in stimulating DNA synthesis in these cultures (approximately 100-fold less effective) while the mitogenicity of bFGF was essentially unchanged.

DISCUSSION

Multiple species of aFGF and bFGF mRNA are present in normal human fibroblasts grown in a serum-free medium that does not contain FGF. Under these conditions, we have demonstrated that these cells produce at least three molecular forms of bFGF protein and three molecular forms of aFGF protein. These studies are the first to demonstrate multiple species of these growth factors in normal human fibroblasts. An accumulation of bFGF protein and bFGF mRNA species were detected after fetal bovine serum treatment. The accumulation of bFGF mRNA was similar (5–10 fold) to that previously reported by our laboratory (26). The abundance of aFGF protein was not affected by serum exposure. Because the method used to detect the FGF proteins was an enzyme-linked immunoblot, it is not possible to obtain an accurate value for the relative levels of aFGF and bFGF proteins produced by these cells. We have not identified the serum factor(s) that are responsible for the increase in bFGF mRNA and accumulation of bFGF protein.

Initial analysis of aFGF and bFGF cDNAs suggested that both of these mitogens are synthesized as 155 AA primary translation products with translation initiated at AUG start codons (1,20). bFGF was originally purified as a 146 AA peptide from bovine pituitary glands, although several other forms of both higher and lower molecular weight have been isolated, all of which have been shown to have the same biological activity as the 146 AA form (10,18,19,21). aFGF protein has been identified in three major forms of 154, 140, and 134 amino acids with corresponding molecular weights ranging from 16 to 18 kD. Proteolytic cleavage during extraction of aFGF from tissue results in a 14 and 20 AA truncation of the 154 AA intact form (13).

Previous studies utilized cDNAs cloned from a human hepatoma cell line to show that CUG (leucine) start codons 5′ to the previously identified AUG start codon (Figure 1) can initiate bFGF translation, and these higher molecular weight forms of bFGF have similar mitogenic activity (7,20). As illustrated in Figure 1, translation initiating from these CUG codons would give rise to peptides of 196 AA, 201 AA, and 210 AA. The higher molecular weight forms of bFGF found in this study (23 and 26.6 kD) could be explained by the utilization of the −122 and −164 CUG codons, which are surrounded by the preferred ribosomal scanning sequences predicted by Kozak (11). In previous studies, Florkiewicz et al. found four sizes of bFGF protein (17, 22.5, 23.1, and 24.2 kD) when analyzing in vitro translation products and protein products of COS 1 cell transfectants. In a similar fashion, Prats et al. found three

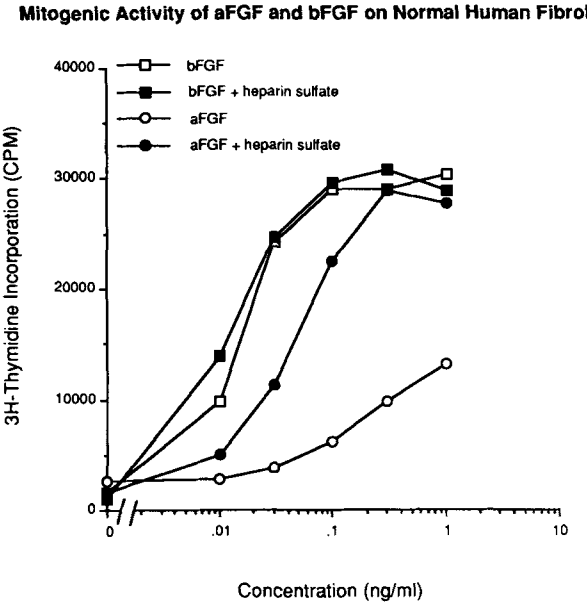

FIG. 6. Response of normal human dermal fibroblasts to varying concentrations of bFGF and aFGF in the presence and absence of heparin sulfate (10 μg/ml). Normal human dermal fibroblasts were plated in MCDB 202a supplemented with 1.0 μg/ml insulin. The cells were treated with the indicated concentrations of either bFGF or aFGF and ^{3}H-thymidine incorporation was determined as described in Materials and Methods.

sizes of bFGF protein; 18, 21, and 22.5 kD. Although the sizes of bFGF protein found in our study were slightly different from the previously reported forms, the differences seem most likely to be due to the use of different gel apparatuses and/or molecular weight markers. Alternatively, multiple forms of bFGF protein could be explained by 5′ sequence heterogeneity among bFGF mRNA species which are the result of differential RNA splicing.

The differential function, if any, of the multiple molecular forms of bFGF remains to be determined. It has been hypothesized that different forms of bFGF protein are produced at different times of the cell cycle and these forms are differentially stored and released in response to stimuli (7). Some of the higher molecular weight forms of bFGF may preferentially migrate to the nucleus of the cell (22).

Our results are the first to show expression of aFGF protein in normal fibroblasts. We detected a 19.2 kD protein which is likely to be the 154 amino acid form of aFGF, and an immunoreactive molecular weight form of aFGF that is larger than the predicted molecular weight of the 154 AA form. It is not known how this putative higher molecular weight form of aFGF protein might arise. Like bFGF mRNA, aFGF mRNA exists as multiple transcripts in human dermal fibroblasts (16). The definitive transcriptional start site for the bFGF and aFGF genes have not been identified. Unlike sequences in bFGF cDNA, the aFGF cDNA contains an in frame translational stop codon three bp 5′ to the putative AUG translation initiation codon as well as potential splice acceptor site 34 bp 5′ to the AUG (16). Recently, in addition to the three previously identified coding exons, two alternative 5′ exons which are utilized to synthesize human aFGF mRNA have been identified (3,5). Crumley et al. found that one of these 5′ exons contains four ATG codons. Thus, it is possible that alternative splicing (3′ to the previously identified AUG) coupled with translation initiating in this newly discovered exon could result in the production of larger molecular weight forms of aFGF protein.

We have shown previously that bFGF and aFGF stimulate multiple rounds of replication in a pooled population of neonatal fibroblasts (24). In the current study, we show that these peptides are synthesized by these cells, thus it is possible that under some conditions the regulation of growth of these cells is under autocrine/paracrine control. Because FGF's effect a broad spectrum of target cells and have numerous biological functions, co-expression of FGF's in fibroblasts may function to coordinate mitogenesis and differentiation of multiple cell types during growth, homeostasis and tissue repair. Neither bFGF nor aFGF have classic signal sequences to target them for secretion, however, several groups have found bFGF in the conditioned media of cultured cells (23,27). We have not detected FGF biological activity in the medium conditioned by human fibroblasts. The question remains how these factors get out of the cell to produce their effects. One hypothesis is that wounding and/or cell death leads to the release of both mitogens into the extracellular space such that binding to cell surface receptors of intact cells could cause growth in a paracrine fashion (14).

In this study, we have shown that bFGF and aFGF proteins are produced by human dermal fibroblasts grown in serum-free medium, and that an accumulation of bFGF protein and mRNA is seen with serum treatment of the cultures. aFGF protein and mRNA production does not appear to be regulated by serum in these cells. Both bFGF and aFGF protein are shown to be present in multiple molecular weight forms. Further studies on the regulation and release of the individual forms of bFGF and aFGF protein will provide insight on their role in both normal and tumor-derived cells.

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References

1. Abraham, J. A.; Whang, J. L.; Tumolo, A., et al. Human basic fibroblast growth factor: nucleotide sequence and genomic organization. EMBO 5(10):2523–2528; 1986.
2. Burgess, W. H.; Maciag, T. The heparin-binding (fibroblast) growth factor family of proteins. Ann. Rev. Biochem. 58:575–606; 1989.
3. Chiu, I. M.; Wang, W. P.; Lehtoma, K. Alternative splicing generates two forms of mRNA coding for human heparin-binding growth factor 1. Oncogene 5(5):755–762; 1990.
4. Cook, P. W.; Coffey, R. J.; Magun, B. E., et al. Expression and regulation of mRNA coding for acidic and basic fibroblast growth factor and transforming growth factor type-alpha in cells derived from human skin. Mol. Endo. 4:1377–1385; 1990.
5. Crumley, G.; Dionne, C. A.; Jaye, M. The gene for human acidic fibroblast growth factor encodes two upstream exons alternatively spliced to the first coding exon. Biochem. Biophys. Res. Comm. 171(1):7–13; 1990.
6. Danielson, P. E.; Forss-Petter, S.; Brow, M. A. p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA 7:261–267; 1988.
7. Florkiewicz, R. Z.; Sommer, A. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc. Natl. Acad. Sci. USA 86:3978–3981; 1989.
8. Goustin, A. S.; Leof, E. B.; Shipley, G. D., et al. Growth factors and cancer. Cancer Res. 46:1015–1029; 1986.
9. Hammond, S. L.; Ham, R. G.; Stampfer, M. R. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc. Natl. Acad. Sci. USA 81:5435–5439; 1984.
10. Klagsbrun, M.; Smith, S.; Sullivan, R., et al. Multiple forms of basic fibroblast growth factor: amino-terminal cleavages by tumor cell- and brain cell-derived acid proteinases. Proc. Natl. Acad. Sci. USA 84:1839–1843; 1987.
11. Kozak, M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell 44:283–292; 1986.
12. Marcis, I.; Adelaide, J.; Raybaud, F., et al. Characterization of the HST-related FGF.6 gene, a new member of the fibroblast growth factor family. Oncogene 4:335–340; 1989.
13. McKeehan, W. L.; Crabb, J. W. Isolation and characterization of different molecular and chromatographic forms of heparin-binding growth factor 1 from bovine brain. Anal. Biochem. 164:563–569; 1987.
14. McNeil, P. L.; Muthukrishnan, L.; Warder, E., et al. Growth factors are released by mechanically wounded endothelial cells. J. Cell Biol. 109:811–822; 1989.
15. Melton, D. A.; Kreig, P. A.; Rebagliati, M. R. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Research 12(18):7035–7056; 1984.
16. Mergia, A.; Tischer, E.; Graves, D., et al. Structural analysis of the gene for human acidic fibroblast growth factor. Biochem. Biophys. Res. Comm. 164(3):1121–1129; 1989.
17. Milner, P. G.; Li, Y. S.; Hoffman, R. M., et al. A novel 17 kD heparin-binding growth factor (HBGF-8) in bovine uterus: purification and N-terminal amino acid sequence. Biochem. Biophys. Res. Comm. 165(3):1096–1103: 1989.

18. Moscatelli, D.; Joseph-Silverstein, J.; Manejias, R., et al. Mr 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA 84:5778–5782; 1987.
19. Moscatelli, D.; Joseph-Silverstein, J.; Presta, M. Multiple forms of an angiogenesis factor: basic fibroblast growth factor. Biochimie 70:83–87; 1988.
20. Prats, H.; Kaghad, M.; Prats, A. C., et al. High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc. Natl. Acad. Sci. USA 86:1836–1840; 1989.
21. Presta, M.; Rusnati, M.; Maier, J., et al. Purification of basic fibroblast growth factor from rat brain: identification of a mr 22,000 immunoreactive form. Biochem. Biophys. Res. Comm. 155(3):1161–1172; 1988.
22. Renko, M.; Quarto, N.; Morimoto, T., et al. Nuclear and cytoplasmic localization of different basic fibroblast growth factor species. J. Cell. Physiol. 144:108–114; 1990.
23. Schweigerer, L.; Neufeld, G.; Friedman, J., et al. Basic fibroblast growth factor: production and growth stimulation in cultured adrenal cortex cells. Endocrinology 120:796–800; 1987.
24. Shipley, G. D.; Keeble, W. W.; Hendrickson, J. E., et al. Growth of normal human keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. J. Cell. Physiol. 138:511–518; 1989.
25. Shipley, G. D. A serum-free [^{3}H]thymidine incorporation assay for detection of transforming growth factors. J. Tiss. Cult. Meth. 10(2):117–124; 1986.
26. Sternfeld, M. D.; Hendrickson, J. E.; Keeble, W. W., et al. Differential expression of mRNA coding for heparin-binding growth factor type 2 in human cells. J. Cell. Physiol. 136:297–304; 1988.
27. Story, M. T. Cultured human foreskin fibroblasts produce a factor that stimulates their growth with properties similar to basic fibroblast growth factor. In Vitro Cell. Dev. Biol. 25(5):402–408; 1989.
28. Thomas, K. A. Fibroblast growth factors. FASEB 1:434–440; 1987.
29. Wang, W.; Lehtoma, K.; Varban, M., et al. Cloning of the gene coding for human class I heparin-binding growth factor and its expression in fetal tissues. Mol. Cell. Biol. 9(6):2387–2395; 1989.