Serum-starvation induces the extracellular appearance of FGF-1

Jordan T. Shin a, Susan R. Opalenik a, John N. Wehby a, Vinit K. Mahesh a, Anthony Jackson b, Francesca Tarantini b, Thomas Maciag b, John A. Thompson a, *

a Division of Transplantation, Department of Surgery, School of Medicine, University of Alabama at Birmingham, UAB Station, Birmingham, AL 35294, USA
b Department of Molecular Biology, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855, USA

Received 13 September 1995; accepted 5 January 1996

Abstract

Autocrine/paracrine stimulation of cell growth by members of the fibroblast growth factor (FGF) family of polypeptides is dependent upon extracellular interactions with specific high affinity receptors at the cell surface. Acidic FGF (FGF-1) lacks a classical signal sequence for secretion, suggesting that intrinsic levels of this mitogen may not stimulate cell growth and utilizes a non-classical pathway to gain access to the extracellular compartment. To evaluate the biological potential of intracellular FGF-1 more rigorously, human cDNA sequences for the growth factor were introduced into primary murine embryonic fibroblasts using retrovirally mediated gene transfer. Heparin affinity, Western analysis, mitogenic assays, in situ immunohistochemical techniques, induction of tyrosine phosphotylation and antibody inhibition studies were used to demonstrate functionality of the FGF-1 transgene in this experimental model. Under normal culture conditions, cells constitutively expressing intracellular FGF-1 exhibited a slight growth advantage. In contrast, when maintained in reduced serum, these cells adopted a transformed phenotype and demonstrated an enhanced growth potential, induction of FGF-specific phosphotyrosyl proteins and the nuclear association of the growth factor. Analysis of the conditioned media from these stressed cells indicated that serum starvation induces the secretion of FGF-1 as latent high molecular mass complexes requiring reducing agents to activate its full biological potential.

Keywords: Serum starvation; Fibroblast growth factor; Biological potential; Primary murine embryonic fibroblast; cDNA sequence

1. Introduction

The prototype members of the fibroblast growth factor (FGF) gene family are well-recognized as initiators of angiogenesis [1,2]. All members of the FGF family share a high degree of sequence similarity at both the nucleotide and amino acid level and have in common several structural motifs. Six members of the human FGF gene family, including FGF-3 (int-2), FGF-4 (hst/KS), FGF-5, FGF-6 (hst-2), FGF-7 (KGF) and FGF-8 (AIGF), each contain an amino terminal secretory signal sequence [3–10] suggesting that autocrine stimulation of cell proliferation by these growth factors requires an extracellular pathway [3–13]. However, FGF-9 (GAF), which does not contain an apparent classical signal sequence, has been shown to be released from cells in vitro [14]. With the exception of FGF-7 and FGF-9, this structural feature correlates with the classification of these FGFs as oncogenes based on their characteristic transforming potential [2].

FGF-1 (acidic) and FGF-2 (basic) are unusual in that, unlike the FGF oncogenes, they lack a classical signal sequence for secretion. This structural feature has made it difficult to evaluate regulation of biological activity by these prototypes. Previous attempts to evaluate the mitogenic potential of FGF-1 and FGF-2 have involved forced secretion of these polypeptides. Transfection [12,15–20] and transduction [21] studies with FGF chimeras containing various signal sequences have described the ability of secreted FGF-1 and FGF-2 to mediate cellular transforma-
tion. Collectively, these and other studies have established that, as extracellular proteins, the biological activity of the FGF prototypes involves productive interactions with a receptor complex on the cell surface [22–24] followed by activation of intrinsic tyrosine kinase [22,25], phosphorylation of specific polypeptides [22], nuclear translocation of the ligand [25,26], and association of the high affinity receptor (FGFR-1) with the nuclear compartment as a structurally intact and functional tyrosine kinase [27].

Previous studies have demonstrated aberrant phenotypic appearance and growth behavior in vitro of cell lines transfected with intrinsic FGF-1 [18–20]. However, these results would seem inconsistent with the fact that proliferation mediated by these widely produced and distributed prototypic growth factors is not observed physiologically, even in those tissues where the concentration of FGF is highest [2]. This suggests that under normal conditions, intracellularly sequestered FGF may not be available to mediate its biological effects on cell growth and differentiation. Consequently, mechanisms whereby the cytosolic growth factors are released into the extracellular environment become fundamental for understanding regulation of FGF biology.

In the absence of defined mechanisms regulating the release of the FGF prototypes from intracellular stores, most studies have proposed that the extracellular appearance of FGF-1 and FGF-2 are the consequence of cell death or damage [28–30]. However, previous results have determined that FGF-2 can be released from cytoplasmic stores by an exocytosis pathway independent of the endoplasmic reticulum (ER)-Golgi apparatus [31]. In addition, FGF-1 has been demonstrated to be released from NIH 3T3 cells in response to heat shock [32] by an ER-independent pathway as a latent complex, which requires activation by reducing agents [33]. More recent efforts [21] have suggested that FGF-1 is secreted from cells as a latent, high molecular mass complex in response to oxidative stress. Since hyperthermic, oxidative stress, and serum-starvation responses overlap [34–37], the role of serum-starvation as a mechanism for inducing the secretion of FGF-1 was examined in vitro. We report that primary embryonic murine fibroblasts, transfected with a single copy of the human FGF-1 gene, are able to release this growth factor in response to serum deprivation. In addition, several lines of evidence suggest that an inverse relationship exists between serum concentration and secretion of FGF-1, which appears in the extracellular compartment as large molecular mass, non-covalent complexes.

2. Materials and methods

2.1. Vector construction

To generate specific retroviral constructs (Fig. 1), the prokaryotic lacZ gene encoding β-galactosidase (β-gal) was cloned into pLNSX [38] following removal of the neomycin phosphotransferase (NPT) gene (Bg). Construction of the synthetic, truncated human FGF-1 gene (amino acids 21–154) has been described and demonstrated to express active recombinant protein in prokaryotic systems [39]. The open-reading frame (ORF) for FGF-1 was cloned 3' of the early SV40 promoter (F) using a synthetic (Applied Biosystems, 380B) linker (27 bp), gatccttccgagtagcgtcagcagccgcccaccATG, which contained sequences for both an untranslated leader (lower case) and an ORF (upper case) that was fused in-frame to the NH2-terminus (ANYKK−) of FGF-1. The untranslated leader (lower case) of the linker included potential ribosome binding site (bold lettering) and Kozak (underlined) sequences for maximal eukaryotic translational efficiency [40–42]. The helper-free packaging cell line GP + envAm12 [43] was cotransfected with either Bg or F together with pCV108, a vector containing the early SV40 promoter directing NPT expression (10:1 molar ratio), using calcium phosphate-mediated precipitation [44]. Cells producing a high titer (106–107 CFU/ml) of amphotropic virus were isolated by P-gal expression using FACS [45] followed by selection (2 to 3 weeks) in 800 μg/ml G418 (Gibco). Viral titration was performed by determining the number of β-gal expressing colonies produced by transductions of 3T3 cells [38] with serial dilutions of supernatant from the producer line. Expression of β-gal was monitored enzymatically [46] with the chromogenic substrate Bluo-gal (Gibco).

2.2. Cell culture, transduction, and selection

Primary murine fibroblasts were isolated from day 12 C57/B6 embryos [47] and established in culture using DMEM (Gibco) supplemented with 10% (v/v) heat-inactivated FBS (Hyclone) and 100 units of penicillin and 100 mg of streptomycin (Gibco). Population doublings were monitored and only early, stabilized passages were
used for these studies. Subconfluent (70–80%) fibroblasts were treated with retroviral particles (moi = 1 to 10) obtained from fresh (6 h) supernatant, conditioned by individual producer lines [21]. Transduced cells were isolated by FACS [45], plated (5 × 10^3 cells/cm²), and expanded in culture for subsequent analyses. Resistance to growth in G418 (500 µg/ml) and lack of a detectable amplification product (PCR) for NPT [48] were used to confirm the absence of packaging cells in individual transduced populations. Routine passage of isolated cells was performed at confluency following 0.05% (w/v) trypsin/0.05 M EDTA, treatment and replating (1 × 10^5 cells/cm²).

2.3. Growth analysis

Transduced cells (1.5 × 10^4) were seeded in 12-well plates (Corning) previously coated with 3 µg/cm² human plasma fibronectin (Gibco) and grown in DMEM supplemented with either 10% (normal serum) or 0.5% (reduced-serum) FBS. Cells were washed (PBS) and fed every three days with fresh media. The number of viable cells (Trypan exclusion) for each transductant were counted by hemacytometer at specific time points, in duplicate, using 5 separate measurements per well. Lactate dehydrogenase (LDH) release assays were used to evaluate cell death/damage according to manufacturer’s specifications (Promega). For a more sensitive assessment of potential sublethal cell injury, cells were incubated (12 mm, 20°C) with a mixture of fluorescein diacetate (FDA, 15 µg/ml) and propidium iodide (PI, 5 µg/ml) in Dulbecco’s-PBS as described [49]. Stained cells were observed immediately by epifluorescence microscopy (Microphot, Nikon) to quantify cell viability. Viable cells were identified by the green yellow fluorescence resulting from hydrolysis of fluorescein diacetate by intracellular esterases. Injured cells were identified by the bright-red fluorescence resulting from the penetration and intercalation of propidium with DNA. The percentage of cell death was computed by assessing the ratio of red staining cells over the sum of red and green staining cells in at least 5 representative fields using a 10 × objective.

2.4. Biochemical analysis

Cells (1 × 10^4/cm²) were seeded and allowed to attach (16 h) under normal growth conditions. Cultures were washed (PBS) and fed DMEM, supplemented with indicated concentrations of FBS for defined periods of time. The simultaneous extraction of DNA and RNA was achieved and analyzed by PCR and RT-PCR techniques using previously established conditions [21]. To obtain extracellular proteins, conditioned media were harvested, centrifuged (2000 × g, 5 min) and filtered (0.45 µ) to remove cellular debris. Total cellular and nuclear proteins were extracted from individual cell populations as described [21]. Total recovered proteins from individual cell populations and compartments were processed separately by affinity extraction using a heparin-Sepharose column [21,39] previously equilibrated in 20 mM Tris-HCl, pH 7.4 containing 1 mM EDTA (TE) using an FPLC system (Pharmacia). Heparin-binding proteins were eluted (2.0 M NaCl in TE) and concentrated/desalted using the Centricon-3 System (Amicon).

2.5. SDS-PAGE and limited SDS-PAGE analysis

Affinity-extracted proteins obtained from both utal cellular and nuclear extracts were fractionated under reducing conditions using routine 15% (w/v) SDS-PAGE [21]. However, total heparin-binding proteins recovered from the conditioned media were resuspended in 62.5 mM Tris-HCl, pH 6.8, containing 5% (v/v) glycerol and 2% (w/v) SDS and immediately subjected to nonreducing 15% (w/v) PAGE analysis under conditions in which neither the stacking and resolving gel, nor the running buffer contained SDS. Gels were transferred electrophoretically to PVDF membranes (Immobilon-P, Millipore) and incubated with an affinity purified antibody (1 µg/ml) against FGF-1, which exhibits no cross-reactivity against FGF-2 and has served as a reagent useful for establishing the involvement of FGF-1 in inflammatory arthritic joints [50]. As a control for FGF-1 monospecificity, the affinity-purified anti-FGF-1 was preincubated (16 h, 4°C) with 100-fold molar excess of recombinant FGF-1 [39], a process which completely blocked immunoblot staining. Recombinant FGF-1 also served as a semi-quantitative immunoreactive marker within the same gel for both SDS-PAGE and limited SDS-PAGE analysis. Membranes were probed with HRP conjugated goat anti-rabbit serum (Kirkegaard and Perry Labs, 1:33 000) and treated with chemiluminescent substrates following manufacturer’s directions (Lumiglo, Kirkegaard and Perry Labs). Films were exposed to luminiscent membranes and developed for varying amounts of time according to manufacturer’s specifications (X-OMAT, Kodak).

2.6. Receptor-mediated tyrosine phosphorylation

To evaluate endogenous levels of tyrosine phosphorylation, cells were seeded (1 × 10^4/cm²) and allowed to attach (16 h) under normal growth conditions. Cultures were washed (PBS) and fed DMEM supplemented with either serum-free, hormonally defined media [20], normal (10%, v/v) or reduced (0.5%, v/v) serum. After 72 h, cells were washed, extracted, immunoprecipitated, and analyzed [21] using polyclonal antibodies against either phosphotyrosine (1 µg/ml), cortactin (1:400) or c-src (1:100, N-16, Santa Cruz). To analyze paracrine stimulation, control (Bg) transduced cells (1 × 10^6) were maintained (48 h) in serum-free, hormonally defined media [20]. Quiescent cells were washed (PBS) and fed (1 h, 37°C) 72-h conditioned media, which was obtained from individual cell popula-
tions maintained under defined culture conditions. Treated cells were washed, extracted, immunoprecipitated, and analyzed [21] using a polyclonal antibody against phospho-
tyrosine (1 μg/ml). Where indicated, conditioned media included pretreatment with 1 mM DTT. Hormonally de-
defined, serum-free media either with or without recombinant FGF-1 (50 ng/ml) and heparin (20 U/ml) served as positive and negative controls (respectively) for these studies.

2.7. In situ immunohistochemical analysis

To measure the proliferation index (PI) of individual transductants, cells (1 · 10^2/cm^2) were seeded and allowed to attach (16 h) under normal growth conditions. Cultures were washed (PBS) and fed DMEM, supplemented with indicated concentrations of FBS for 48 h followed by labeling for 4 h with bromodeoxyuridine (BrdU, Sigma, 10 μM). Where indicated, conditioned media contained 30 μg/ml of an anti-FGF-1 polyclonal antibody (Sigma) added at 0, 24 and 48 h time points. To validate the mitogenic potential of the affinity-extracted FGF-1 transgenes, NIH 3T3 cells (1 · 10^4/cm^2) were seeded and allowed to attach (16 h) under normal growth conditions. Cultures were washed (PBS) and maintained (36 h) in 0.5% (v/v) FBS. Either recombinant FGF-1 or the FGF-1 transgenes, heparin-extracted from intra- and extracellular compartments, were added (20 h) in the presence of heparin (5 U/ml) to quiescent cells followed by labeling (4 h) with BrdU. Where indicated, recombinant FGF-1 and affinity-extracted transgenes were preincubated (16 h, 4°C) with 30 μg/ml polyclonal anti-FGF-1 antibody (Sigma) prior to addition. Activation of the high molecular mass FGF-1 complexes, extracted from the extracellular compartment, was achieved by pretreatment with 1 mM DTT. Harvested cells were resuspended (10^5 cells/ml) and 0.1 ml aliquots were centrifuged (Cytospin, Shandon-Lipshaw) onto glass microscope slides and immediately fixed, washed and analyzed as described [21]. Immunohistochemical localization of FGF-1 was performed using Vectastain Elite ABC following manufacturer’s recommendations (Vector Labs). Cells were centrifuged, fixed and depleted of endogenous peroxidase activity. Fixed cells were washed and incubated (30 min) with 1.5 μg/ml affinity-purified anti-FGF-1 [50]. Primary murine fibroblasts transduced [21] with a retroviral vector engineered to direct expression of (hst/KS) FGF-1, a secreted, chimeric form of FGF-I, were used as a positive control. Negative controls included incubation of fixed cells with preimmune rabbit serum (10%) and deletion of the primary antibody. An additional control for FGF-1 specificity included antibody blocking conditions as described for Western analyses, which completely blocked cellular staining. Antibody-treated samples were incubated with biotinylated secondary antibody (goat, anti-rabbit) followed by avidin-biotin HRP (Vector ABC). Chromogenic development of stained samples permitted analysis via light microscopy. Nuclear-associated staining was determined by dividing DAB-stained nuclei by the total observed nuclei and statistical significance was assessed as described [21], using Student’s t-test (Abacus Concepts, Berkeley, CA).

3. Results

Primary cultures of embryonic murine fibroblasts were transduced independently with retroviral vectors (Fig. 1), selected by FACS, and expanded for biochemical analyses. The transduction efficiency, as determined by percentage of cells expressing Bg (FACS), were approximately the same for each retroviral vector (30–35%). No attempt was made to subclone specific cell populations relative to expression levels of either the selectable marker (Bg) or modulatory transgene (FGF-1). Characteristic PCR (DNA) and RT-PCR (RNA) products for Bg (355 bp) and the transduced FGF-1 (390 bp) were identified in expanded populations of individual cell transductants (data not shown). These results were consistent with the engineered retroviral nucleic acid sequences, thereby confirming both delivery of the nucleic acid sequences and the ability of primary murine fibroblasts to coordinate a transcriptional response from these templates. Similar levels of both Bg and FGF-1 transgene mRNA were identified (RT-PCR) in individual cell transductants, regardless of culture conditions (10%, v/v, or 0.5%, v/v, FBS). Translation of Bg mRNA was confirmed on expanded populations of trans-
duced cells using both analytical FACS and routine enzym-
atic analyses. Greater than 98% of cells in each trans-
duced population produced readily detectable levels of active Bg.

When compared to Bg-transduced cells, cells constitutively expressing intrinsic FGF-1 exhibited a slight growth advantage under normal (10%, v/v, FBS) conditions (Fig. 2A). When maintained in reduced serum (0.5%, v/v, FBS), Bg-transduced cells demonstrated minimal growth during the 12 days in culture. However, growth analysis of FGF-1-transduced cells demonstrated a similar initial quies-
cent state for 6 days followed by a significantly in-
creased proliferative potential (Fig. 2B). When maintained in reduced serum (0.5%, v/v, FBS), both Bg- and FGF-1-
transduced cells released minimal levels of LDH into the extracellular compartment. Normalization of LDH units to total cell number determined that 1.94% of Bg-transduced cells experienced cell death/damage when maintained 72 h in reduced serum. This value was reduced significantly (P < 0.01) to 1.37% in FGF-1-transduced cells. Assessment of FDA-PI staining following experimental conditions of serum deprivation determined that 3.17% of Bg-
transduced cells demonstrated potential sublethal cell in-
jury compared (P < 0.02) to 1.08% observed in FGF-1-
transduced cells. Assessment of Trypan blue exclusion correlated well with both these assays and confirmed the
growth potential of FGF-1 transgenes: (A) Under growth arrest conditions (72 h; 0.5%, v/v, FBS), the percentages of Bg- and FGF-1-transduced cells (1×10^4/cm^2) labeled (BrdU incorporation, 4 h) were determined in the presence (•) or absence (○) of daily (72 h) additions of 30 µg/ml anti-FGF-1 polyclonal antibody (Sigma). (B) Heparin Sepharose-extracted recombinant FGF-1 (20 ng/ml) or FGF-1 transgenes (approx. 10–15 ng/ml), recovered from either the intracellular or extracellular compartment of FGF-1-transduced cells maintained (72 h) in reduced (0.5% (v/v) FBS) or reduced (0.5% (v/v) FBS) serum, respectively, were analyzed for their ability to stimulate DNA synthesis in quiescent NIH 3T3 cells. Mitogenic activity (labeling index) was determined by BrdU incorporation in the presence (•) or absence (○) of 30 µg/ml polyclonal anti-FGF-1 antibody (Sigma), preincubated (16 h, 4°C) with the affinity-extracted FGF-1 transgene. Activation of the extracellular complex was achieved by pretreatment with 1 mM DTT prior to addition or preincubation with the antibody.

For a more detailed analytical evaluation, growth potentials were evaluated by determining the proliferation index (BrdU incorporation) of individual transduced cell populations maintained (3 days) under defined culture conditions (Fig. 3A). The percentages of Bg- and FGF-1-transduced cells labeled under normal conditions (10%, v/v, FBS) conditions were very similar (40% and 43%, respectively) and did not demonstrate a significant statistical difference. Under growth arrest conditions (0.5%, v/v, FBS), the labeling index of control-transduced cells (Bg) was reduced to 2.6%. In contrast, FGF-1-transduced cells retained a high proliferation index (10.9%) in reduced serum that was significantly (P < 0.01) enhanced when compared to Bg-transduced cells. Daily additions (30 µg/ml) of polyclonal antibody against FGF-1 under conditions of growth arrest (3 days; 0.5%, v/v, FBS) had no effect on Bg-transduced cells. In contrast, the polyclonal anti-FGF-1 significantly (P < 0.01) reduced the proliferation index (5.5%) of FGF-1-transduced cells maintained in reduced serum.
The morphology of individual cell populations, seeded (1 \( \cdot \) 10\(^4\)/cm\(^2\)) and maintained (72 h) in normal serum (10\%, v/v, FBS) conditions, were observed microscopically. Both Bg- and FGF-1-transduced cells displayed (Fig. 4A and B) a typical monolayer phenotype throughout the time course of study. In contrast, under identical seeding densities (1 \( \cdot \) 10\(^4\)/cm\(^2\)), FGF-1- but not Bg-transduced cells adopted a transformed cellular phenotype (Fig. 4C), characterized by focal growth patterns and a spindle-like morphology, when maintained (72 h) in reduced serum (0.5\%, v/v). This observation is consistent with previous transfection studies using FGF chimeras containing various signal sequences [18–21] which have described the ability of secreted FGF-1 to mediate cellular transformation. In addition, daily feeding fresh media (0.5\%, v/v, FBS) to the FGF-1-transduced cells retarded their growth rate and prevented the appearance of the transformed phenotype. Bg-transduced cells adopted this characteristic transformed phenotype when treated daily for 3 days with recombinant FGF-1 (50 ng/ml) alone or in combination with heparin (20 USP units/ml). The morphological response to these daily treatments was dependent on constant presentation of the growth factor since its elimination resulted in reversion to a normal phenotype.

Induction of cellular transformation by recombinant FGF-1 treatments and antibody inhibition (Fig. 3A) of the proliferation index of FGF-1-transduced cells suggested the potential involvement of this growth factor during phenotypic changes induced by serum-starvation. Consequently, in situ immunohistochemical methods were used to evaluate expression of FGF-1 in individual transductants. Under normal culture conditions (10\%, v/v, FBS),

![In situ immunohistochemical analysis of transduced cells. Sub-confluent populations of FGF-1-(panels A, C and D) and (hus/kS) FGF-1- [21] (panel B) transduced cells were maintained (72 h) in either 10\% (v/v) FBS (panels A and B) or 0.5\% (v/v) FBS (panels C and D). Cytospin preparations of transduced cells were analyzed by immunohistochemical techniques using the affinity-purified polyclonal antibody specific for FGF-1 [50] and localization of the growth factor was visualized (brown staining) by light microscopy (240 \times magnification). Note absence of nuclear-associated staining in FGF-1-transduced cells maintained (72 h) in 0.5\%, v/v, FBS containing daily additions (0, 24, 48 h) of 30 \( \mu \)g/ml polyclonal anti-FGF-1 antibody (panel D).](image-url)
FGF-1-transduced cells readily demonstrated increased immunoreactivity for FGF-1. Staining was distributed largely within the cytoplasmic compartment with occasional arrays confined to the plasma membrane (Fig. 5A). In contrast, FGF-1-transduced cells maintained in reduced serum (0.5%, v/v, FBS) displayed immunoreactivity for FGF-1, not only distributed within the cytosol but also associated with the nuclear compartment in 28.9% of the total cell population (Fig. 5C). Daily additions of antibody against the polypeptide growth factor significantly (P < 0.001) inhibited the appearance of immunoreactivity (< 5.2% total cells) associated with the nucleus (Fig. 5D). As a positive control, cells transduced with a secreted, chimeric form of FGF-1, exhibited immunoreactivity for FGF-1 localized predominantly in the nuclear compartment of greater than 95% of the cell population (Fig. 5B).

Biochemical analyses also were performed on individual cell populations under defined culture conditions. Western blot analysis demonstrated that minimal levels (approximately 4 ng/10^6 cells) of affinity-extracted, immunoreactive protein (17 kDa) were present in total cellular extracts from both transduced cell populations suggesting that embryonic fibroblasts express low, but detectable levels of the full length, endogenous murine FGF-1 (Fig. 6A–C, lane 2). Expression of the truncated FGF-1 transgene (Fig. 6B and C, lane 2) was limited to FGF-1-transduced cells, which demonstrated steady-state levels of the intracellular growth factor (approximately 40–80 ng/10^6 cells) that were unaffected by culture conditions (10%, v/v, or 0.5%, v/v, FBS). Preincubation (16 h, 4°C) of the antibody with 100-fold molar excess of recombinant FGF-1 completely blocked Western blot staining. In addition, Western analysis of total, unfractonated cell extracts failed to detect the presence of any immunoreactive bands. It should be noted, that in our experience, techniques of PAGE and efficient electrophoretic transfer are limited to 50 100 μg of total protein per loading. This value represents less than 1 × 10^3 total cell extract which represents a level of the FGF-1 transgene below 8 ng. a value representing the lower limits of detection by the Western techniques used in this study. Consequently, affinity-extraction (heparin-Sepharose) techniques permitted the opportunity to analyze FGF-1 proteins in a more rational manner.

The truncated FGF-1 transgene, affinity-extracted from the total intracellular compartment of FGF-1-transduced cells, displayed mitogenic behavior in the DNA synthesis assay similar to that obtained with recombinant FGF-1 isolated from E. coli (Fig. 3B). Pretreatment of both these growth factor preparations with the antibody against FGF-1 significantly (P < 0.001) inhibited their mitogenic potential. Correlation of this biological data with immunoblot analysis determined that the intracellular form of FGF-1 retained greater than 90% of its mitogenic potential. The presence of the affinity-extracted, truncated FGF-1 transgene (approximately 20–30 ng/10^6 cells) in nuclear extracts (Fig. 6, lane 3) was restricted to FGF-1-transduced cells maintained in reduced serum (0.5%, v/v, FBS, 72 h). These results complement in situ immunohistochemical analysis (Fig. 5) and predict the extracellular appearance of FGF-1, since nuclear localization involves an exogenous pathway [26].

Western analysis of concentrated media (10% or 0.5%, v/v, FBS) alone or conditioned (72 h) by Bg-transduced cells failed to demonstrate the presence of extracellular FGF-1, even when affinity-extracted with heparin-Sepharose (Fig. 6A). In contrast, FGF-1-transduced cells maintained (72 h) in reduced serum demonstrated the extracellular presence of immunoreactive FGF-1 migrating primarily as multiple high molecular mass bands, which were resolvable by limited SDS-PAGE immunoblot analysis and included a large percentage of material displaying an apparent molecular mass of approx. 32 kDa (Fig. 6B and C, lane 6). Pretreatment of the affinity-purified antibody with recombinant FGF-1 (100-fold molar excess) pre-
Fig. 7. Kinetics of FGF-1 secretion in response to serum-starvation. Affinity extracted proteins recovered from media conditioned by FGF-1-transduced cells (1 x 10⁶) were fractionated by non-reducing 15% (w/v) Limited SDS-PAGE and evaluated by Western analysis using a polyclonal antibody specific for FGF-1. (Panel A) The extracellular appearance of immunoreactive FGF-1 was analyzed as a function of time in culture. Lanes 2, 3, 4, 5 and 6 correspond to proteins recovered following 24, 48, 60, 72 and 84 h, respectively, of exposure to 0.5% (v/v) FBS. (Panel B) The extracellular appearance of immunoreactive FGF-1 was analyzed as a function of serum concentration in the culture media. Lanes 2, 3, 4, 5 and 6 correspond to proteins recovered from FGF-1-transduced cells maintained (72 h) in 10, 5, 2, 0.5 and 0.2% (v/v) FBS, respectively. Recombinant FGF-12154 (0.3 μg; lane A and B) and FGF-11154 (0.4 μg; lane 7, A and B) served as controls. Approximate sizes (kDa) were estimated using molecular mass standards (right).

Fig. 8. Autocrine/paracrine stimulation of tyrosine phosphorylation. Individual cell extracts were immunoprecipitated with a monoclonal anti-phosphotyrosine antibody, resolved by either 8% w/v (A and B) or 12% w/v (C) reducing SDS-PAGE, and Western analyzed with polyclonal antibodies against either phosphotyrosine (A and B) or c-src (C). (Panel A) Total endogenous cellular extract immunoprecipitated from FGF-1-transduced cells (1 x 10⁶) maintained (72 h) in either serum-free, hormonally defined media (lane 1), 0.5% (w/v) FBS (lane 2), or 10% (w/v) FBS (lane 3). (Panel B) Total cellular extracts immunoprecipitated from quiescent Bg-transduced cells (5 x 10⁶) following treatment (37°C, 60 min) with media (0.5% (w/v) FBS) conditioned (72 h) by 1 x 10⁶ Bg-transduced cells (lane 1), untreated (lane 2) or treated (1 mM DTT, lane 3) media conditioned for 72 h (0.5% (v/v) FBS) by 1 x 10⁶ FGF-1-transduced cells, or heparin (20 U/ml) and 10 ng/ml recombinant FGF-12154 (lane 4). (Panel C) Total endogenous cellular extract immunoprecipitated from FGF-1-transduced cells (1 x 10⁶) maintained (72 h) in either 10%, v/v (lane 1) or 0.5% (v/v) FBS (lane 2). Arrowheads (left) identify responding phosphotyrosyl polypeptides, whose approximate sizes (kDa) were estimated using prestained molecular mass standards (right).
wherein maximal levels were achieved within 60 h and persisted throughout the time course of study (84 h). Additional efforts included Western analysis of heparin-extracted media conditioned by FGF-1-transduced cells maintained for 72 h in different concentrations of serum (Fig. 7B). These results demonstrate that the extracellular appearance of the FGF-1 complexes is inversely related to the concentration of serum in the culture media, wherein maximum levels were observed in 0.5% (v/v) and 0.2% (v/v) FBS (Fig. 7B, lanes 5 and 6, respectively). Limited SDS-PAGE analysis failed to demonstrate the presence of extracellular, immunoreactive FGF-1 as a native structure during these kinetic studies.

To validate further the biological potential of extracellular FGF-1, endogenous levels of tyrosine phosphorylation were examined in FGF-1-transduced cells maintained (72 h) under defined culture conditions (Fig. 8A). When compared to cells quiesced in serum-free, hormonally defined media, FGF-1-transduced cells grown in complete media (10%, v/v, FBS) demonstrated a slight increase in tyrosine phosphorylation levels (Fig. 8A, lane 3). In contrast, FGF-1-transduced cells that were maintained in reduced serum (0.5% FBS, v/v) demonstrated an exaggerated tyrosine phosphorylation of polypeptides with apparent molecular masses of 150, 130 and 90 kDa (Fig. 8A, lane 2). Also, when compared to cells maintained in 10% (v/v) FBS, increased phosphorylation of endogenous c-src, migrating with an apparent molecular mass of approx. 60 kDa, was evident in FGF-1-transduced cells maintained (72 h) in reduced (0.5%, v/v, FBS) serum (Fig. 8C). Increased tyrosine phosphorylation of endogenous cortactin, a substrate for c-src [51], also was observed as a consequence of culturing FGF-1-transduced cells in reduced serum (not shown). In addition, reduced-serum media conditioned (72 h) by FGF-1-transduced cells induced FGF-specific tyrosine phosphorylation in quiescent Bg-transduced cells (Fig. 8B, lane 2), an observation that was exaggerated by pretreatment of the conditioned media with DTT (Fig. 8B, lane 3). Similar results were obtained following pretreatment of this conditioned media with N-acetyl cysteine and reduced, but not oxidized, glutathione (not shown). Phosphorylation of tyrosine residues on polypeptides with apparent molecular masses of 150, 130 and 90 kDa (Fig. 8B, lane 4) is consistent with those substrates responding to exogenous recombinant FGF-1 [22,23] and correlates with the requirement for the presence of reducing agents to activate the mitogenic potential (Fig. 3B) of latent, extracellular FGF-1 released from FGF-1-transduced cells in response to serum deprivation.

4. Discussion

A wide variety of diploid cells either in vitro or in vivo both express and respond to FGF-1, a prototypic member of the FGF family of polypeptide growth factors that does not contain a classical signal peptide sequence for secretion. This feature alone suggests conserved evolutionary pressure against the extracellular presentation of this growth factor, which would complete an autocrine/paracrine loop leading to inopportune biological consequences. Evidence of this potential has been demonstrated in vivo wherein the delivery of a single dose of exogenous FGF-1 induced site-specific angiogenesis and neurogenesis in adult animals [52,53]. Similar results have been obtained in porcine arteries following introduction of a DNA vector orchestrating expression of a secreted form of FGF-1 [54]. In addition, the ability of FGF-1 to disrupt the normal pattern of lens differentiation in transgenic mice is restricted to the extracellular presentation of this polypeptide growth factor [55]. Consequently, mechanisms whereby FGF-1 gains access to the extracellular compartment are requisite to understanding the involvement of this mitogen in pathophysiologic situations, normal physiologic repair mechanisms, and regulation of development.

Most diploid cells in vitro, including primary embryonic murine fibroblasts, express relatively low levels of endogenous FGF-1, suggesting that large scale cell culture would be required to study trafficking and compartmentalization of the growth factor. To overcome this limitation, retroviral vectors were designed to deliver a single copy of the human cDNA sequence, which would direct constitutive overexpression of intracellular FGF-1 as a biologically active protein and thereby permit evaluation of growth factor partitioning in normal diploid fibroblasts under defined, small-scale culture conditions. The truncated form of the growth factor was chosen both to allow discrimination between the full length of endogenous murine and the transduced human gene product and to prevent potential translation problems previously encountered in E. coli [39]. As an extracellular protein, this form of FGF-1 has been demonstrated to retain full biological activity both in vitro [39] and in vivo [52–55]. Results presented here are consistent with these observations and further suggest that deletion of the first 21 amino acids from the NH₂-terminus generates a form of the growth factor that retains full biological activity including structural features related to heparin and receptor binding [56], nuclear localization [25], cytosolic retention [33], and extracellular release as a function of biological stress [32,33].

Several features of the experimental model used in this study provide insight into the physiological consequences of intrinsic FGF-1 expression beyond that previously reported in transfection experiments, which have documented the ability of intrinsic FGF-1 to mediate cellular transformation in established cell lines [18–20]. In contrast to these studies, primary murine fibroblasts constitutively expressing FGF-1 under normal culture conditions maintained both a representative cellular phenotype and an anchorage-dependency for growth. It is suggested that, compared to transfected established cell lines, transduced primary cultures of normal cells reflect a situation more
reminiscent of the in vivo environment by maintaining stringent control over growth promoting processes, including mechanisms that regulate the retention, secretion and biological consequences of intracellular FGF-1. High titer supernatants generated transduction efficiencies that permitted immediate isolation (FACS), expansion, and biochemical analyses of transduced primary cells, which were subjected to minimal population doublings and non-stressful culture densities. Less efficient transfection experiments typically require cells to survive both long-term selection in culture and growth under stressful, low density conditions. Consequently, aberrant phenotypic appearance and growth behavior in vitro of cell lines transfected with intrinsic FGF-1 would be consistent with the release of the growth factor from cells in response to biological stress [32].

Initial evidence of an extracellular pathway for release of FGF-1 included the observation that FGF-1-transduced cells maintained in normal culture conditions (10%, v/v, FBS) demonstrated a slight growth advantage when compared to control-transduced cells. Under optimal conditions of growth, FGF-1-transduced cells also exhibited a slight increase of endogenous FGF-specific tyrosine phosphorylation which correlated with low, but detectable levels of extracellular FGF-1. Interestingly, the use of a novel immunoblot assay was able to detect the secreted form of FGF-1 as multiple, high molecular mass complexes, which were readily dissociated with reducing agents to a single species of representative molecular mass. Under normal culture conditions, FGF-1 was not detected as a nuclear-associated protein in FGF-1-transduced cells; however, this may reflect the sensitivity limits of both the immunoblot and the in situ techniques used in these studies. Collectively, these results suggest that FGF-1-transduced primary murine fibroblasts contain an autocrine/paracrine loop operating at either a reduced or minimal level under conditions requisite for cell growth.

In contrast to cells constitutively expressing intrinsic FGF-1 under normal culture conditions, FGF-1-transduced cells maintained in reduced serum demonstrated: (i) an increased growth advantage with delayed kinetics, (ii) retention of a high proliferation index that was inhibited by inclusion of the anti-FGF-1 antibody in the culture media, (iii) a change in cellular morphology to a more transformed phenotype, (iv) the appearance of immunoreactive FGF-1 associated with the nuclear compartment by a process inhibited by extracellular antibody against FGF-1, and (v) an increase of endogenous FGF-specific tyrosine phosphorylation. Collectively, these observations predict the induction of an FGF-1 extracellular autocrine/paracrine loop in response to serum deprivation. As an isolated result, increased levels of endogenous tyrosine phosphorylation do not rule out the potential existence of other polypeptide factors in the conditioned media which may stimulate converging signal transduction cascades. However, under conditions of serum deprivation, we have not identified the extracellular appearance of FGF-2, which has been demonstrated to be secreted from cytoplasmic stores by a nonconventional pathway [31]. The mitogenic potential of FGF-1 involves nuclear association of the polypeptide by an extracellular pathway that can occur either through high affinity receptors and associated signal transduction events [26] or by transport of the polypeptide into the cell in the absence of tyrosine phosphorylation [57]. Results here are consistent with the former mechanism, wherein serum starvation induced both nuclear association of FGF-1 and FGF-specific tyrosine phosphorylation, including increased phosphorylation of the FGF receptor-1 substrate, c-src [51] and cortactin, a substrate for c-src which responds to exogenous FGF-1 [58]. Consequently, it seemed reasonable to anticipate the appearance of the growth factor in reduced-serum media conditioned by FGF-1-transduced cells. Indeed, when compared to normal culture conditions, FGF-1-transduced cells exposed to reduced serum demonstrated release of increased levels of the multiple, high molecular mass complexes, which were readily dissociated to a biologically active, native form of FGF-1 by treatment with DTT. This observation suggests that serum-starvation induced a nonconventional pathway for secretion of FGF-1, an interpretation which is consistent with that observed during temperature-induced [32,33] release of the growth factor.

The kinetics of the extracellular appearance of FGF-1 in response to serum deprivation were evaluated to characterize the secretory process in vitro. The cellular release of FGF-1 complexes was inversely related to serum concentration with maximum levels of secretion requiring greater than 48 h exposure to 0.5% (v/v) FBS. The growth potential, proliferation index, appearance of a transformed phenotype, induction of FGF-specific phosphorylation, and nuclear association of FGF-1 correlated with the kinetics of FGF-1 secretion. Differential levels of gene expression did not contribute to either the delayed kinetics or the overall levels of growth factor secretion since similar levels of FGF-1 mRNA and protein were observed in the FGF-1-transduced cells maintained in either 10% (v/v) or 0.5% (v/v) FBS. Assessment of both LDH release and FDA-PI staining suggested that sublethal cell injury did not contribute to the release of FGF-1. In fact, when compared to Bg-transduced cells, serum-deprived populations of FGF-1-transduced cells demonstrated significantly reduced levels of cell death/damage. This observation alone suggests the induction of a biological mechanism to promote cell survival.

Whereas the exact identity of the extracellular, high molecular mass FGF-1 complexes are not known, our data are consistent with the recent observations that FGF-1 is released in response to heat shock [32] and HIV-1 TAT protein [21] as a biologically inactive homodimer. Indeed, the major species detected in our immunoblot assay corresponds well with the reported molecular mass representative of the FGF-1 homodimer induced by copper oxidation.
As a homodimer structure, FGF-1 is biologically inactive and requires reduction of oxidized cysteine residues to restore complete heparin affinity, receptor binding and full mitogenic potential [59]. The ability of reducing agents to both dissociate the secreted FGF-1 complexes, activate the full mitogenic potential of extracellular FGF-1, and exaggerate paracrine stimulation of FGF-specific tyrosine phosphorylation is consistent with these results. Collectively, these observations, coupled with the involvement of FGF-1 cysteine residues during temperature-induced release of the growth factor [33], suggest a common FGF-1 secretory pathway. While the exact biological mechanisms responsible for formation and activation of the secreted FGF-1 complex are not known, we suggest that localized redox potential may be involved, particularly since oxidative and hypothermic stress responses overlap [34]. The observation that continuous (daily) feeding of FGF-1 transduced cells prevented the induction of FGF-1 specific receptor-mediated cellular responses is consistent with this concept, since the process will not only remove extracellular FGF-1 complexes but also restore intracellular and extracellular redox potentials [60]. Results presented here suggest further that media conditioned by FGF-1 transduced cells contain an appropriate mechanism to activate some of the latent FGF-1 complexes, thereby permitting completion of an extracellular autocrine/paracrine loop. The absence of detectable steady state levels of extracellular FGF-1 as a native structure, the appearance of characteristics associated with exogenous FGF-1 treatment, and the observation that native FGF-1 requires only partial receptor occupancy to initiate DNA synthesis in vitro [52] would be consistent with this interpretation. However, the rate of complex activation (reduction) appears to be reduced compared to that of formation-secretion as evidenced by the accumulation of high molecular mass FGF-1 species and the observed delayed kinetics associated with growth in reduced serum.

An underlying implication of our studies with serum deprivation, a situation more reflective of the in vivo environment, suggests that the nonconventional pathway for FGF-1 secretion occurs at a reduced level under normal physiologic conditions. This feature would confirm a potential mechanism whereby reducing agents may act as nutrients to promote low density cell growth in vitro [61] and provide biological insight into the process whereby FGF-1 functions as a survival factor [62]. Increased growth factor secretion observed in cells constitutively expressing high levels of FGF-1 mRNA would be consistent with the suggestion that regulation of this nonconventional pathway is controlled by levels of newly translated polypeptide rather than by activation of cytosolic stores [33]. It remains to be determined whether secretion and activation of latent FGF-1 complexes are involved during in vivo pathophysiologic conditions, such as atherosclerosis [63] and kidney injury [64], which have been demonstrated to include increased transcription of FGF-1 mRNA.

Acknowledgements

The authors thank S. Nan, X. Liu, Q. Li and M. L. Spell for excellent technical assistance; B. Johnson for expert technical assistance; and R. Friesel, Holland Laboratory, American Red Cross, for the polyclonal anti-phosphotyrosine. Submitted in partial fulfillment of the requirements for a doctorate of philosophy from the Departments of Biochemistry/Molecular Genetics (J.T.S.) and Medical Genetics (S.R.O.), University of Alabama at Birmingham. This work was supported in part by NIH grants HL32348 and HL44336 to T.M., HL08391 to V.K.M., and HL45990 and HL48491 to J.A.T. Synthesis of oligonucleotide linkers and amplimers was supported by NCI Grant CA13148 to the UAB Comprehensive Cancer Center.

References