Recovery of Mitogenic Activity of a Growth Factor Mutant with a Nuclear Translocation Sequence

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Heparin-binding growth factor–1 (HBGF-1) is an angiogenic polypeptide mitogen for mesoderm- and neuroectoderm-derived cells in vitro and remains biologically active after truncation of the amino-terminal domain (HBGF-1α) of the HBGF-1β precursor. Polymerase chain reaction mutagenesis and prokaryotic expression systems were used to prepare a mutant of HBGF-1α lacking a putative nuclear translocation sequence (amino acid residues 21 to 27; HBGF-1U). Although HBGF-1U retains its ability to bind to heparin, HBGF-1U fails to induce DNA synthesis and cell proliferation at concentrations sufficient to induce intracellular receptor–mediated tyrosine phosphorylation and c-*fos* expression. Attachment of the nuclear translocation sequence from yeast histone 2B at the amino terminus of HBGF-1U yields a chimeric polypeptide (HBGF-1U2) with mitogenic activity in vitro and indicates that nuclear translocation is important for this biological response.

THE HEPARIN-BINDING (FIBROblast) growth factor (HBGF) family of polypeptide mitogens includes two prototypic, three oncogenic, and two additional polypeptide members that are structurally related (*1, 2*). Whereas most of these polypeptides have been characterized as mitogens for various mesoderm- and neuroectoderm-derived cells, the prototypes, HBGF-1 (acidic FGF) and HBGF-2 (basic FGF) are of interest because their precursor structures do not contain signal sequences for secretion (*1*). Although the biological significance of this structural feature remains unclear, the precise cellular locale of the HBGF prototype translation products in vivo has been a subject of considerable interest (*3*).

Immunohistochemical localization studies with human rheumatoid and osteoarthritic tissue revealed that HBGF-1 stained intracellularly within blood vessels, bone, and synovial tissue in vivo (*4*). As observed previously for HBGF-2 with endothelial cells (*5*), immunohistochemical staining within mesenchymal cells demonstrated nuclear localization of HBGF-1 (*4*). Because nuclear translocation of polypeptides has a structural basis (*6, 7*), we examined the HBGF family for consensus sequences described for polypeptides translocated to the nucleus (*7*). Interestingly, HBGF-1 contained the sequence NYKKPKL (residues 21 to 27, Fig. 1 (*8*), which is similar to nuclear translocation sequences of other nuclear proteins (*7*). Therefore, we constructed and expressed an HBGF-1 deletion mutant lacking residues 1 to 27 of the precursor polypeptide HBGF-1β (Fig. 1) and examined the biological activity of the recombinant translation product.

We first constructed a synthetic gene for HBGF-1α in which the amino acid sequence was divided into four domains. Oligonucleotides encoding each domain were

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                  1        10        20        30        39
HBGF-1β   MAEGEITTFTALTEKFNLPPGNYKKPKLLYCSNGGHFLRI
HBGF-1α             MANYKKPKLLYCSNGGHFLRI
HBGF-1U                       MLYCSNGGHFLRI
HBGF-1U2            MGKKRKSKAKMLYCSNGGHFLRI
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Fig. 1. Structure of the NH_2-terminal domain for the HBGF-1 precursor and mutants. The amino acid sequence of residues 1 to 39 for the HBGF-1 precursor, HBGF-1β; the amino acid sequence of the NH_2-terminal truncation, HBGF-1α; the NH_2-terminal deletion mutant, HBGF-1U; and the amino acid sequence of the chimera, HBGF-1U2, containing the aligned NH_2-terminus of HBGF-1U ligated to the nuclear translocation sequence of yeast H2B. Acidic FGF, another NH_2-terminal truncated derivative of the HBGF-1β precursor, begins at phenylalanine-15 (*29*).

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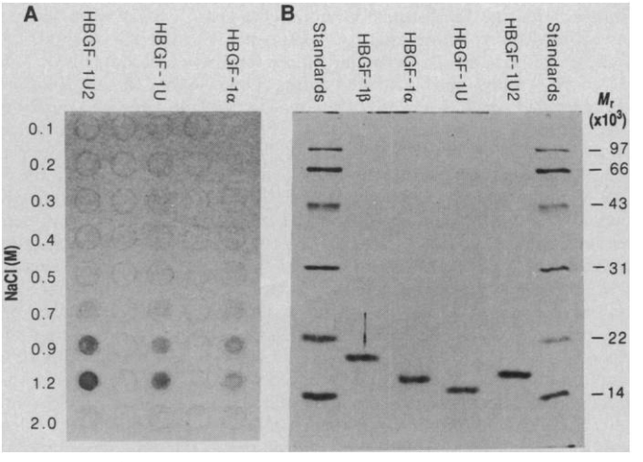

Fig. 2. Prokaryotic expression of the NH_2-terminal deletion mutants and chimeras of HBGF-1. (**A**) Heparin affinity: The BL21(DE3) pLysS cells containing the synthetic constructs for either HBGF-1α, HBGF-1U, or HBGF-1U2 in the pET-3c vector (*11*) were cultured in 2 ml of LB medium containing ampicillin (50 μg/ml) and chloramphenicol (40 μg/ml). At late log phase of growth, expression of the individual recombinant proteins was induced with 0.5 mM isopropylthiogalactoside for 2 hours at 37°C. The cells were collected by centrifugation, and suspended in 1 ml of 25 mM tris-HCl (pH 7.2), containing 10 mM EDTA and 50 mM glucose. The cells were freeze-thawed twice, sonicated, and allowed to undergo lysis with their endogenous lysozyme. The supernatant was adjusted to 0.1 M NaCl, mixed with 50 μl of heparin-Sepharose beads, and incubated at 4°C for 2 hours on a rotating platform. The heparin-Sepharose beads were centrifuged, washed three times each with 1 ml of TE buffer [10 mM tris-HCl (pH 7.2), containing 1 mM EDTA], and were sequentially washed with TE buffer containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 0.9, 1.2, or 2.0 M NaCl. Eluate samples (50 μl) were blotted onto a nitrocellulose membrane, and the recombinant HBGF-1 polypeptides were visualized with the use of a rabbit antibody to HBGF-1 in combination with alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin G (IgG) (Promega). (**B**) Purification of the HBGF-1 mutants: The proteins were expressed as described in (A), and lysates from a 1-liter culture were adjusted to 0.5 M NaCl in TE buffer. The lysate was incubated with 5 ml of heparin-Sepharose and, after washing with 0.5 M NaCl in TE buffer, the beads were packed into columns, washed extensively with 0.7 M NaCl in TE buffer, and the HBGF-1 polypeptides were eluted from the columns with 1.2 M NaCl in TE buffer. The proteins were further purified on a Vydac C4 reversed-phase high-pressure liquid chromatography (HPLC) column as described (*9*). The recombinant proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (15% polyacrylamide gel) and stained with Coomassie blue. Amino acid analysis and NH_2-terminal microsequencing (25 cycles) were performed with each recombinant polypeptide for quantitative protein analysis and to verify the sequence of each mutant. Because the purity of each recombinant HBGF-1 polypeptide was greater than 90% after the heparin affinity step, we used these polypeptides for the biological experiments.

synthesized, ligated, and cloned into individual cassettes, and the four cassettes ligated to form the complete open-reading frame (ORF) for HBGF-1α (*9*). Because the putative nuclear translocation sequence resided at the NH_2-terminal end of HBGF-1α and because the biological activities of the HBGF-1β precursor and HBGF-1α were very similar (*10*), we used the synthetic gene encoding the HBGF-1α ORF as a template for polymerase chain reactions (PCRs) to enzymatically generate inserts for HBGF-1U and HBGF-1U2 (Fig. 1), using synthetic oligonucleotide primers specific for each mutant (*11*). The nucleotide sequence for each mutant was confirmed and each synthetic ORF translated in the T7 RNA polymerase expression system (*11*).

The first mutant polypeptide examined was HBGF-1U, lacking the putative nuclear localization sequence, NYKKPKL (Fig. 1). In that HBGF-1U was able to bind immobilized heparin, (Fig. 2A) with an affinity similar to that described for HBGF-1α (*10, 12*), we were able to purify HBGF-1U from *Escherichia coli* translation lysates (Fig. 2B). Confirmation of the deletion within HBGF-1U was determined by NH_2-terminal microsequencing (*13*). To examine the biological activity associated with HBGF-1U, we used a [^{3}H]thymidine incorporation assay (*10*), as well as a cell proliferation assay (*12*), and showed that HBGF-1U was unable to induce either DNA synthesis or cell growth (Fig. 3, B to D). Assays performed in the presence of heparin yielded similar results (Fig. 3, C and D). Because (i) exogenous HBGF-1 exerts a mitogen response by the occupancy of a high-affinity receptor (*14*), (ii) HBGF-1 receptor binding stimulates tyrosine phosphorylation of membrane-bound and cytosolic polypeptides (*15*), and (iii) the structure of the HBGF-2 receptor contains a c-*fms*–like intracellular tyrosine kinase domain (*16*), we examined the ability of HBGF-1U to induce tyrosine phosphorylation. Indeed, immunoprecipitation with antibodies to phosphotyrosine demonstrated that HBGF-1U, like HBGF-1α (*15*), was able to induce the phosphorylation of four polypeptides (Fig. 4) at concentrations where HBGF-1U did not stimulate cell proliferation and DNA synthesis in vitro (Fig. 3, B to D). These data suggested that although HBGF-1U was not able to initiate either DNA synthesis or cell division, the mutant could induce the tyrosine-specific phosphorylation of membrane-associated (150-, 130-, 90-, and 40-kD) polypeptides. We next examined the ability of HBGF-1U to induce the expression of c-*fos* mRNA, an immediate early response gene for HBGF-1α (*17*); indeed, HBGF-1U induced the expression of c-*fos* mRNA (Fig. 5). These data suggested that (i) the phosphorylation of the receptor on tyrosine residues and the stimulation of c-*fos* expression is insufficient for the stimulation of DNA synthesis, and (ii) the mitogenic defect with HBGF-1U may be due to the failure of the mutated polypeptide to undergo nuclear translocation.

We reasoned that if nuclear translocation was important for mitogenic activity of HBGF-1, it may be possible to restore the mitogenic activity to HBGF-1U by the addition of a well-recognized nuclear translocation sequence. Therefore, we constructed a chimeric polypeptide (HBGF-1U2, Fig. 1) containing the HBGF-1U sequence with the yeast histone 2B (H2B) nuclear translocation sequence (GKKRKSKAK) at the NH_2-terminus (*18, 19*). Indeed, the recombinant chimeric polypeptide, HBGF-1U2 (Fig. 1), that contained the yeast H2B nuclear translocation sequence, retained its ability to bind heparin (Fig. 2, A and B) and possessed mitogenic activity in vitro (Fig. 3, C and D). In the absence of heparin, HBGF-1U2 was as potent as the recombinant HBGF-1α polypeptide in its ability to initiate DNA synthesis and to stimulate

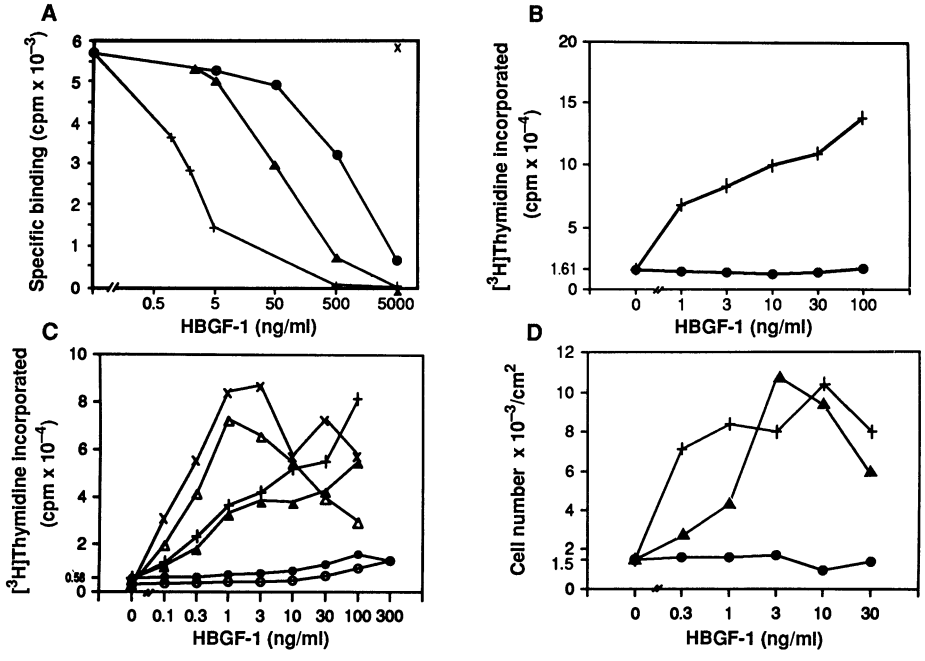

Fig. 3. Biological activity of the HBGF-1 mutants. (**A**) Competitive receptor binding. Bovine HBGF-1α was ^{125}I-labeled with immobilized lactoperoxidase and purified by heparin-Sepharose as described (*12*). Confluent murine lung capillary endothelial cells (LE-II) were serum-starved for 4 hours before binding experiments with Dulbecco's modified Eagle's medium (DMEM) containing 0.5% fetal bovine serum (FBS). The cells were washed and incubated with Hanks' balanced salt solution containing heparin (5 units/ml), 1% bovine serum albumin (BSA) in 20 mM Hepes, pH 7.2 (binding buffer) at room temperature for 20 min. The cultures were then transferred onto ice, and the cells were exposed to ^{125}I-labeled HBGF-1α (2 ng/ml) with the indicated concentrations of competitor HBGF-1 or BSA (control). After incubation at 4°C for 2 hours with rocking, the cells were washed three times with binding buffer and solubilized with 0.2 M NaOH. Radioactivity was measured with a gamma counter. Nonspecific binding (bound ^{125}I-labeled HBGF-1α in the presence of HBGF-1α at 5 μg/ml) was subtracted and did not exceed 20% of total binding. All the values were determined from at least duplicate samples. (**B**) DNA synthesis by LE-II cells. Confluent LE-II cells were in DMEM, containing 10% FBS, and were serum-starved for 2 days in DMEM containing 0.5% charcoal-absorbed FBS. The test samples were added to the media at the indicated concentrations, and the cells were cultured at 37°C. After 18 hours, [^{3}H]thymidine was added to a final concentration of 1 μCi/ml. After 4 hours, the cells were washed with phosphate-buffered saline (PBS), treated with 10% trichloroacetic acid (TCA), solubilized with 0.2 M NaOH, neutralized with 0.2 M HCl, and the radioactivity was quantitated by liquid scintillation. All values were determined in triplicate. (**C**) DNA synthesis by 3T3 cells. NIH 3T3 2.2 cells (*14*) were brought to confluency in DMEM, containing 10% calf serum, and starved for 2 days as above. The DNA synthesis assay was similar to the assay described for LE-II cells, except that the samples were added either without or with heparin (HP) at 5 units/ml. All values were determined in triplicate. (**D**) Human endothelial cell growth. Confluent human umbilical endothelial (HUVE) cells were trypsin-treated, grown on plates coated with 5 μg of human fibronectin in Medium 199 containing 10% FBS at a cell density of 2.5×10^3 per square centimeter as described (*9*), and allowed to attach at 37°C for 9 hours. The test samples were then added to the indicated final concentration with heparin at 5 units/ml (day 0) with media change on day 2 and 4. On day 5, the cells were detached with trypsin, and viable cell numbers were quantitated with a hemacytometer. All values were determined by duplicate samples except the HBGF-1–free control, where six samples were quantitated.

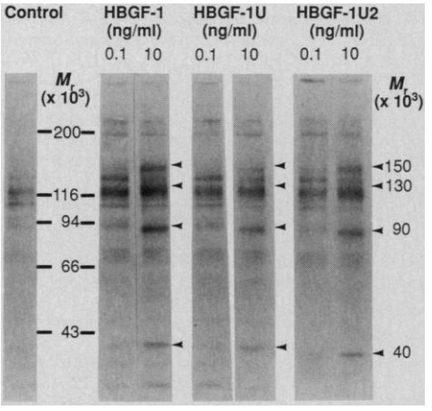

Fig. 4. The induction of tyrosine phosphorylation by the HBGF-1 mutants. Confluent, serum-starved NIH 3T3 2.2 cells were metabolically labeled for 3 hours at 37°C with 0.33 mCi of [^{32}P]orthophosphate per milliliter in 25 mM Hepes (pH 7.4), containing 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, and 0.1% BSA. The cells were exposed to 0, 0.1, or 10 ng of HBGF-1α, HBGF-1U, or HBGF-1U2 per milliliter for 10 min at 37°C. The cells were washed once with cold PBS (pH 7.4), containing 50 mM NaF, 30 mM sodium pyrophosphate, and 100 μM sodium orthovanadate and lysed in buffer containing 10 mM tris-HCl (pH 7.4), containing 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1.0% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer). The cells were immediately scraped from the plates, spun in a vortex mixer, and incubated on ice for 10 min. Lysates were centrifuged at 10,000*g* for 10 min at 4°C. Supernatants were incubated for 2 hours at 4°C with constant rotation, with 20 μl of a 50% suspension of monoclonal antibody to phosphotyrosine coupled to Sepharose. The Sepharose beads were washed four times with lysis buffer, and [^{32}P]phosphotyrosine-containing proteins were specifically eluted with 10 mM phenyl phosphate in 10 mM tris-HCl (pH 7.4), 50 mM NaCl, 0.1% Triton X-100, and 0.1% ovalbumin. Phosphoproteins were analyzed by SDS-PAGE (7.5% polyacrylamide) and visualized by autoradiography. HBGF-1–stimulated proteins of 150-, 130-, 90-, and 40-kD are shown. Phosphoamino acid analysis of the 150-, 130-, and 90-kD proteins have confirmed phosphorylation on tyrosine (*15*).

human endothelial cell division (Fig. 3, B to D). In addition, the ability of heparin to potentiate the biological activity of HBGF-1, a feature that distinguishes between the HBGF prototypes (*20*), was also reconstituted by the addition of the yeast H2B nuclear translocation sequence (Fig. 3, C and D). Further, HBGF-1U2, like HBGF-1α and HBGF-1U, was able to initiate the phosphorylation of the membrane-associated (150-, 130-, 90-, and 40-kD) polypeptides on tyrosine residues (Fig. 4) and to induce the expression of c-*fos* mRNA (Fig. 5). Murine 3T3 cells transfected with HBGF-1U2 and HBGF-1α assumed a conventional transformed cellular phenotype and were able to proliferate under serum-free conditions in vitro. In contrast, 3T3 cells transfected with HBGF-1U did not assume a transformed phenotype and exhibited a serum dependency for cell proliferation (*21*). Although it was also possible to detect HBGF-1α and HBGF-1U2 in the nucleus of transfected cells, it was not possible to identify intranuclear HBGF-1U within transfectants, even though all three forms of HBGF-1 were readily detected in the cytosol of the individual transfectants (*21*). In addition, the intracellular fate of HBGF-2 has been determined by analysis of HBGF-2 transfection, and considerable HBGF-2 has been observed within the nucleus of 3T3 cells transfected with the HBGF-2 cDNA that encodes the larger molecular weight form of HBGF-2 (*22*). Recent evidence also suggests that translocation of HBGF-2 to the nucleus of bovine endothelial cells is cell cycle–dependent and occurs in the late G_1 phase of the cell cycle (*23*) and site-directed mutagenesis of HBGF-1 at lysine-132 yields a recombinant HBGF-1 mutant polypeptide with biological properties similar to those described for HBGF-1U (*24*).

Although the mitogenic activities of HBGF-1α and HBGF-1U2 were similar, HBGF-1U2 was less efficient by a factor of about 10 to 25 than HBGF-1α in displacing ^{125}I-labeled HBGF-1α from its receptor (Fig. 3A). Similar data were also obtained with competitive ligand-receptor covalent cross-linking methods (*25*). HBGF-1α and HBGF-1U2 at similar concentrations not only stimulated HBGF receptor–mediated tyrosine phosphorylation and c-*fos* expression, but HBGF-1U2 also induced these responses at concentrations that were weakly competitive with ^{125}I-labeled HBGF-1α for receptor binding (Figs. 3A and 4A). These results suggest that the presence of the putative nuclear translocation sequence may override deficiencies in HBGF-1U2 receptor binding, and thus, may enable relatively small concentrations of the polypeptide to initiate DNA synthesis and cell growth. However, HBGF-1U was less efficient by a factor of about 80 to 100 than HBGF-1α in competition with ^{125}I-labeled HBGF-1α for its receptor (Figs. 3A), but required only a tenfold excess of polypeptide to initiate tyrosine phosphorylation (Fig. 4B) and c-*fos* expression (Fig. 5). Thus, HBGF-1U may contain sufficient structural information to mimic the ability of HBGF-1α to initiate tyrosine phosphorylation and c-*fos* expression, but only at a concentration that is weakly competitive with ^{125}I-labeled HBGF-1α for receptor binding. These data are consistent with earlier observations that partial HBGF-1 receptor occupancy is sufficient to initiate DNA synthesis in murine endothelial cells in vitro (*14*). However, our data do not eliminate the possibilities that HBGF-1U (i) is less stable than HBGF-1α and HBGF-1U2, (ii) fails to recognize an intracellular intermediate important for the regulation of DNA synthesis, or (iii) induces a level of tyrosine phosphorylation and c-*fos* expression that is insufficient for the induction of cell division.

The ability to delete and reconstitute the mitogenic activity of HBGF-1α by the manipulation of a putative nuclear translocation sequence argues that translocation of HBGF-1 to the nucleus may be necessary for the stimulation of DNA synthesis in vitro. Nuclear translocation sequences have also been found in the two homodimer forms of platelet-derived growth factor (PDGF-A and PDGF-B) (*18*), potent polypeptide mitogens for many mesenchymal cell types (*26*), and the PDGF-B translocation product has been found in the nucleus of simian sarcoma virus–transformed cells (*27*). In addition, interleukin-1 (IL-1), a polypeptide regulator of immune function and inflammation (*28*) that has no signal

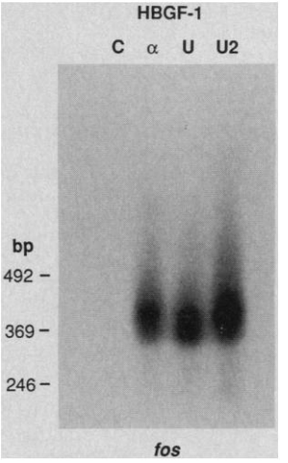

Fig. 5. The induction of c-*fos* transcription by the HBGF-1 mutants. Confluent murine 3T3 2.2 cells were serum-starved as described in the legend to Fig. 3B for 2 days. The cells were treated with 10 ng of HBGF-1α, HBGF-1U, or HBGF-1U2 per milliliter and incubated at 37°C. After 15 min, the cells were washed twice with PBS, and total RNA was prepared as described (*17*). One microgram of RNA from each sample was reverse-transcribed with c-*fos* antisense primer (5′-CAAAGCAGACTTCTCATCTTC-3′). The cDNA was then amplified by the PCR (*21*) with both antisense and sense (5′-TGGCCGTCTCCAGTGCCAACTT-3′) primers (*33*). The amplified products were run on a 1.3% agarose gel, transferred to a nitrocellulose membrane, and visualized with a c-*fos* DNA probe radiolabeled by random priming followed by autoradiography. The band of the amplified product c-Fos (380 bp) is indicated by the arrow. Molecular size markers are shown to the left of the figure.

sequence and that is structurally related to the HBGF prototypes (*29*), has also been found to be transported to the nucleus (*30*). Indeed, a putative nuclear translocation sequence has been identified in IL-1α (*30*); the sequence, NYPKKKMEKR, is present in IL-1β (*31*). Also the structure of an IL-1–like polypeptide containing a signal sequence has been recently described (*32*). Further, intracellular IL-1α has been implicated in the regulation of human endothelial cell senescence in vitro (*33*). Thus, these data imply that HBGF-1 may ultimately act as an intracellular, nuclear-translocated polypeptide mitogen, a feature that would not require a signal sequence.

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8. Single-letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Extension of the Life-Span of Human Endothelial Cells by an Interleukin-1α Antisense Oligomer

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The proliferative potential of human diploid endothelial cells is finite, and cellular senescence in vitro is accompanied by the failure of the endothelial cell to respond to exogenous growth factors. Senescent human endothelial cells were shown to contain high amounts of the transcript for the cytokine interleukin-1α (IL-1α), a potent inhibitor of endothelial cell proliferation in vitro. In contrast, transformed human endothelial cells did not contain detectable IL-1α messenger RNA. Treatment of human endothelial cell populations with an antisense oligodeoxynucleotide to the human IL-1α transcript prevented cell senescence and extended the proliferative lifespan of the cells in vitro. Removal of the IL-1α antisense oligomer resulted in the generation of the senescent phenotype and loss of proliferative potential. These data suggest that human endothelial cell senescence in vitro is a dynamic process regulated by the potential intracellular activity of IL-1α.

THE NUMBER OF CELL DIVISIONS that human diploid fibroblasts undergo in vitro is both finite and a function of the number of cumulative population doublings (*1*). The lifetime of human cells under controlled conditions in vitro is reproducible and is inversely proportional to the in vivo age of the donor (*2*). Although a variety of theories have been proposed to explain the phenomenon of cellular senescence in vitro, evidence suggests that the age-dependent loss of proliferative potential may be genetically programmed (*3*). Cell fusion studies with human fibroblasts in vitro have demonstrated that the quiescent cellular senescent phenotype is dominant over the proliferative phenotype (*4*) and that protein synthesis in senescent cells, before fusion with young cells, is required for the inhibition of DNA synthesis within the young nucleus of the heterodikaryon (*5*). Likewise, the microinjection of senescent fibroblast mRNA into young fibroblasts inhibits the ability of the young cell to synthesize DNA (*6*) and inhibits the entry of

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