# Extracellular Matrix Contains Insulin-like Growth Factor Binding Protein-5: Potentiation of the Effects of IGF-I

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*Abstract.* Insulin-like growth factor binding proteins (IGFBPs) have been shown to serve as carrier proteins for the insulin-like growth factors (IGFs) and to modulate their biologic effects. Since extracellular matrix (ECM) has been shown to be a reservoir for IGF-I and IGF-II, we examined the ECM of cultured human fetal fibroblasts and found that IGFBP-5 was incorporated intact into ECM, while mostly inert proteolytic fragments were found in the medium. In contrast, two other forms of IGFBP that are secreted by these cells were either present in ECM in minimal amounts (IGFBP-3) or not detected (IGFBP-4). Likewise, when purified IGFBPs were incubated with ECM, IGFBP-5 bound preferentially. IGFBP-5 was found to bind to types III and IV collagen, laminin, and fibronectin. Increasing salt concentrations inhibited the binding of IGFBP-5 to ECM and accelerated the release of IGFBP-5 from ECM, suggesting an ionic basis for this interaction. ECM-associated IGFBP-5 had a sevenfold decrease in affinity for IGF-I compared to IGFBP-5 in solution. Furthermore, when IGFBP-5 was present in cell culture substrata, it potentiated the growth stimulatory effects of IGF-I on fibroblasts. When IGFBP-5 was present only in the medium, it was degraded to a 22-kD fragment and had no effect on IGF-I–stimulated growth. We conclude that IGFBP-5 is present in fibroblast ECM, where it is protected from degradation and can potentiate the biologic actions of IGF-I. These findings provide a molecular explanation for the association of the IGF's with the extracellular matrix, and suggest that the binding of the IGF's to matrix, via IGFBP-5, may be important in mediating the cellular growth response to these growth factors.

P EPTIDE growth factors have typically been studied by determining the response of cells to soluble growth factors that diffuse rapidly through the culture medium. However, in vivo, connective tissue cells are imbedded in a complex extracellular matrix (ECM)[1], and diffusible growth factors may be exposed to the extracellular matrix (ECM) before binding to receptors (29, 35). Access to the receptor may involve intermediate binding of the growth factor to components of the matrix. For example, attachment of growth factors such as transforming growth factor-$\beta$ (TGF-$\beta$) and basic fibroblast growth factor (bFGF) to matrix components can serve to stabilize or increase local concentrations of the growth factors (12), to modulate the interaction of these peptides with their receptors (44, 45), to alter their rates of diffusion through the matrix (33), or to protect them from proteolytic degradation (36). As a result, the cellular response to growth factors that are bound to the pericellular ECM can be markedly different than the response to the same peptides in solution.

Both insulin-like growth factors (IGF-I and IGF-II) have been shown by immunohistochemical staining to localize to specific cell types within tissues (18, 24) that do not contain their mRNAs, as determined by in situ hybridization (13). In contrast, the cells where the IGFs are concentrated do synthesize IGF binding proteins (IGFBPs). Therefore, IGFBPs have been postulated to transport and direct the IGFs to specific cell types within tissues (8). Previous studies have also demonstrated that IGFBPs bind to cell surfaces and modulate the binding of IGF-I to its receptors (9). The IGFs are known to localize in ECM (14), and IGF-II was purified from bone cell matrix (27). However, studies to determine if IGFBPs are present in ECM have not been reported. Recently, we have observed that fibroblasts synthesize three distinct forms of IGFBP: IGFBP-3, IGFBP-4, and IGFBP-5 (7). The purposes of the current study were to identify which, if any, of these IGFBPs were present in fibroblast ECM, to compare their relative amounts, to measure the affinity of the ECM-associated IGFBPs for the IGFs, and to determine whether ECM-localized EGFBPs modulate cellular growth responses to IGF-I.

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## Materials and Methods

### Preparation of Extracellular Matrix

Human fetal dermal fibroblasts (GM10; NIGMS Human Genetic Mutant Cell Repository, Camden, NJ) were maintained in Eagle's Minimal Essential Media (EMEM; Hazelton Systems, Denver, PA) supplemented with 10% calf serum (Colorado Serum Co., Denver, CO) and 110 $\mu$g/ml pyruvate, 30 $\mu$g/ml asparagine, 21 $\mu$g/ml serine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. For experiments in which it was desired to minimize the amount of IGFBP-5 deposited into the ECM, the cells were maintained in 10% FBS (Hyclone Laboratories, Logan UT) rather than calf serum. The cells were plated on positively charged 24- or 96-well plates (Primaria, Falcon Laboratory Division of Becton Dickinson, Rutherford, NJ) and grown for 7-10 d until confluence. The Primaria plates were used to minimize nonspecific binding of IGFBP-5 to the dishes. ECM was prepared as described by Knudsen et al. (23), keeping the plates on ice and using ice-cold solutions. The cells were rinsed twice in PBS and the cell membranes removed by incubation for 10 min in 0.5% Triton X-100 in PBS, pH 7.4. The adherent nuclei and cytoskeleton were removed by incubation for 5 min in 25 nM ammonium acetate, pH 9.0. The ECM, which remained on the plates, was rinsed twice in PBS and used as substratum for binding or growth experiments, for immunocytochemistry or for Western blot analysis.

### Western Ligand Blots and Immunoblots

Conditioned media were collected from confluent cultures of fibroblasts that had been rinsed twice and incubated in serum-free EMEM (250 $\mu$l/cm$^2$) containing 0.01% BSA for 48 h. Fibroblast ECM was prepared as above and scraped from the plates in Laemmli sample buffer (25) containing 2% SDS. Samples of the ECM preparations and conditioned media were heated to 60°C in sample buffer for 10 min, and solubilized proteins were resolved by SDS-PAGE in 12.5% gels and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp., Bedford, MA). The membranes were probed with $^{125}$I-IGF-I using a ligand blotting technique previously described (19), or with a 1:1,000 dilution of a polyclonal antiserum to human IGFBP-5 (7). After overnight incubation at room temperature in the IGFBP-5 antiserum, the immunoblots were developed by incubating 2 h with a anti-guinea pig IgG-HRP conjugate (Sigma Immunochemicals, St. Louis, MO). A chemiluminescent peroxidase substrate (ECL; Amersham, Arlington Heights, IL) was applied according to the manufacturer's instructions and the membrane was exposed briefly to autoradiographic film (Hyperfilm-MP; Amersham Corp., Arlington Heights, IL).

### Immunocytochemistry

ECM from human fetal fibroblasts was prepared as described above, except on glass microscope slides adapted for cell culture (Lab-Tek Chamber slides; Nunc, Naperville, IL). The ECM on the slides was fixed with 10% formaldehyde/45% acetone in 200 mg/l Na$_2$HPO$_4$ and 1 g/l KH$_2$PO$_4$, pH 7.2, for 1 min, rinsed in TBS (10 mM Tris-HCl, pH 7.5, 150 mm NaCl) and blocked for 60 min with 3% BSA in TBS with 0.05% NaN$_3$. The slides were rinsed with TBS and incubated overnight at 4°C with 1:1,000 dilutions (in TBS with 1% BSA) of either control nonimmune sera, primary antisera, or primary antisera that had been preincubated with excess antigen. The primary polyclonal antisera used were directed against human fibronectin, laminin, vitronectin, decorin, tenascin (Telios Pharmaceuticals, San Diego, CA), against human IGFBP-1 (6), bovine IGFBP-2 (10), recombinant human IGFBP-3, or human IFGBP-5 (7). After rinsing with TBS, the slides were incubated for 2 h with 1:50 dilutions of monoclonal anti-rabbit IgG (monoclonal anti-guinea pig IgG was used for the IGFBP-5 primary antiserum) conjugated to biotin (Sigma Chemical Co.), followed by incubation with avidin-biotin-HRP complex (Elite ABC Kit; Vector Labs, Burlingame, CA) according to the manufacturer's directions. The antibody-bound peroxidase was visualized by incubation with 3,3-diaminobenzidine in the presence of nickel and hydrogen peroxide (DAB substrate kit; Vector Labs). The stained ECM preparations were sealed on the slides with Crystal/Mount (Biomeda, Foster City, CA) and mounted under coverslips with PerMount (Sigma Chemical Co.). The slides were examined and photographed under light microscopy.

### Preparations of Human IgF Binding Proteins

Human IGFBP-5 was purified to homogeneity from the conditioned media of human glioblastoma cells (T98G; American Type Culture Collection, Rockville, MD) as previously described (7). The protein was proven to be pure IGFBP-5 by NH$_2$-terminal amino acid sequence analysis, which showed that the first 30 residues were identical to the published sequence (39). Human IGFBP-5 was also prepared from media conditioned by CHO cells (CHO-K1; American Type Culture Collection) that had been stably transfected with an expression vector containing a human IGFBP-5 cDNA, the expression of which was driven by a mouse metallothionein promoter. Isolation of the IFGBP-5 cDNA from T98G human glioblastoma cells by polymerase chain reaction has been described (7). This cDNA was inserted into the pNUT plasmid expression vector (obtained from Dr. R. Palmiter, University of Washington, Seattle, WA; 30) as previously described for IGFBP-1 (20). The recombinant CHO protein was purified as previously described (7) and was indistinguishable from the IGFBP-5 purified from glioblastoma conditioned medium by SDS-PAGE with silver stain, ligand blot, and immunoblot analysis or by IGF binding affinity determination. Recombinant human IGFBP-3 synthesized in transfected CHO cells was a generous gift of Genentech, Inc. (South San Francisco, CA). Human IGFBP-1 was purified from amniotic fluid (5), bovine IGFBP-2 from Madin Darby bovine kidney cells (3), and human IGFBP-4 from T98G glioblastoma conditioned media (7) as previously described.

### Attachment and Release of IGFBP-5 from Matrix

To measure the binding of soluble IGFBP-5 to ECM, the ECM was prepared from GM10 fibroblasts grown on Primaria (positively charged) 24-well plates. Primaria plates were used to minimize nonspecific binding of IGFBP-5 to the plastic. (These plates coated with IGFBP-5 bound <3% total $^{125}$I-IGF-I added as compared to 15% for negatively charged culture plates.) The cells had been maintained in 10% FBS to minimize the presence of endogenously synthesized IGFBP-5 (see Fig. 1). The ECM-coated wells were incubated with 100 ng/ml IGFBP-5 in 0.25 ml of either a buffer containing 10 mM NaH$_2$PO$_4$, 10 mM Na$_2$HPO$_4$, pH 7.4, 0.1% BSA (30 mM Na$^+$), or the same buffer to which additional NaCl had been added, to final Na$^+$ concentrations of 75 mM, 150 mM, 300 mM, 600 mM, or 1.0 M. After an overnight incubation at 4°C the wells were rinsed twice, and the ECM-attached IGFBP-5 was extracted with Laemmli sample buffer containing 2% SDS and detected by SDS-PAGE and ligand blot analysis using $^{125}$I-IGF-I. To examine the release of IGFBP-5 from ECM, the ECM was prepared from GM10 fibroblasts maintained on 24-well plates in 10% calf serum to maximize the amount of endogenous IGFBP-5 incorporated into the ECM. The wells were rinsed twice and incubated with 0.25 ml of the same buffers (containing 0.03-1.0 M sodium) used for the binding experiment. After 2.5- and 5-h incubations at 22°C, the ECM was extracted with SDS and the remaining IGFBPs detected by ligand blot analysis, as described above for the binding experiment. In addition, IGFBP amounts in the releasates were determined by adding Tween-20 to a final concentration of 0.05%, concentrating 10-fold with a Centricon-10 (Amicon Corp.) microconcentrator and adding NaCl to equalize the final sodium concentrations of all samples to 1.0 M. The concentrated samples were analyzed by SDS-PAGE and ligand blot and immunoblot analysis, and IGFBP in the samples quantitated by an IGF-I binding assay performed as described previously (5).

The rate of loss of endogenous IGFBP-5 from ECM was determined by incubating ECM (prepared from GM10 fibroblasts cultured in 10% CS on 96-well tissue culture plates) in 0.1 ml serum-free EMEM with 0.01% BSA at 37°C. The amount of IGFBP-5 remaining in the ECM after various incubation times was determined by ligand blot analysis. The rate of loss of exogenous IGFBP-5 was determined by first allowing purified IGFBP-5 (80 ng/ml) to attach to ECM (prepared from GM10 fibroblasts cultured in 10% FCS to minimize endogenous IGFBP-5) by overnight incubation at 4°C in 0.1 ml 30 mM NaH$_2$PO$_4$, pH 7.4, with 0.1% BSA. The ECM preparations were then rinsed twice and the rate of loss of IGFBP-5 from the ECM (at 37°C in EMEM with 0.01% BSA) was determined as described above for endogenous IGFBP-5. The binding affinity of the exogenous IGFBP-5 released into 50 $\mu$l of releasate during a 6-h incubation period was measured in a solution $^{125}$I-IGF-I binding assay (performed at pH 6.0) as described previously (20), to determine whether it had been denatured as a result of matrix association.

### Binding of Purified IGFBP-5 to Individual Components of ECM

Individual matrix components were purchased from commercial sources: type I collagen (rat tail; Sigma Chemical Co.); types III, IV, and V collagen

(human placenta; Sigma Chemical Co.); type VII collagen (human fetal membranes; Telios); laminin (Englebreth-Holm-Swarm mouse sarcoma; Sigma Chemical Co.); vitronectin (human plasma; Telios); and cellular fibronectin (human foreskin fibroblasts; Sigma Chemical Co.). The collagens were dissolved in 0.1 M acetic acid to 10 $\mu$g/100 $\mu$l, and 100 $\mu$l per well was applied to 96-well plates (Corning #25805-96; Corning Inc., Corning, NY) in triplicate and allowed to dry. Laminin, vitronectin, and fibronectin were diluted to 10 $\mu$g/100 $\mu$l in PBS, and 100 $\mu$l per well were applied to the wells in triplicate, incubated 3 h at 22°C, and aspirated. Each well was rinsed twice with PBS and incubated overnight at 4°C with 50 ng of IGFBP-5 in 100 $\mu$l of 30 mM NaH$_2$PO$_4$, pH 7.4, with 0.1% BSA. Each well was then rinsed twice with PBS and the attached proteins solubilized and removed with Laemmli sample buffer containing 2% SDS. The attached IGFBP-5 was detected by SDS-PAGE of the solubilized proteins, followed by transfer to polyvinylidene difluoride membranes and ligand blotting with $^{125}$I-IGF-I.

## Binding of IGF-I to IGFBP-5 and IGFBP-3 Attached to ECM or to Collagen

ECM from human fetal fibroblasts was prepared as described above from cells maintained in 10% FBS on 24-well Primaria tissue culture plates. Collagen-coated wells were prepared by incubating 24-well Primaria tissue culture plates with 20 $\mu$g of human placental type IV collagen (Sigma Chemical Co.) in 100 $\mu$l 0.1 M acetic acid and allowing the collagen solution to dry. The ECM- and collagen-coated wells were rinsed twice with binding buffer (EMEM without bicarbonate, supplemented with 20 mM Hepes, pH 7.4, and 0.1% BSA) and incubated overnight at 4°C in 0.25 ml with the same binding buffer and 100 ng/ml of either IGFBP-5, IGFBP-3, or with buffer alone (controls). The wells were then rinsed twice in binding buffer and incubated for 4 h at 22°C and 20,000 cpm of $^{125}$I-IGF-I (a generous gift of Dr. Louis Underwood, University of North Carolina, Chapel Hill, NC) and 0-32 ng/ml unlabeled IGF-I in 0.25 ml of the same buffer. Some release of IGFBP-5 from the ECM occurred in 4 h at 22°C in physiologic salt (see Fig. 3 b). After rinsing with PBS, the bound radioactivity was solubilized with 0.3 M NaOH and counted in a $\gamma$-spectrometer. Binding of $^{125}$I-IGF-I directly to uncoated or collagen-coated Primaria plates was <1% of the total radioactivity when the $^{125}$I-IGF-I was added before and <3% when it was added after the plates were incubated with IGFBP-5. In contrast, incubation of IGFBP-5 with uncoated (negatively charged) tissue culture plates resulted in binding of 15% of total $^{125}$I-IGF-I. Nonspecific binding of the $^{125}$I-IGF-I to matrix or matrix components was determined by coincubation with 1,000 ng/ml recombinant IGF-I (a generous gift of Genentech), was consistently <5% of total cpm added. Specific binding to the endogenous IGFBPs in the ECM (which was minimized by culturing the cells in FBS and was usually <20% of binding detected after exposure to IGFBP-5) was subtracted from total binding at each competing concentration of IGF-I.

## Binding of IGF-I to IGFBP-5 in Solution

Competitive binding assays using $^{125}$I-IGF-I (20,000 cpm) and unlabeled IGF-I (0-8 ng/ml) with IGFBP-5 (16 ng/ml) were performed in solution in 0.25 ml of the same binding buffer used for the ECM binding experiments at pH 7.4. Bound and free IGF-I were separated by precipitation in 12.5% polyethylene glycol (PEG-8000; Sigma Chemical Co.) as described previously for IGFBP-1 (26). Nonspecific binding was determined by competition with 1,000 ng/ml unlabeled IGF-1, was <25% of B$_0$, and was subtracted.

## Fibroblast Growth Assay

To determine the effects of ECM and IGFBP-5 on fibroblast growth, GM10 fetal fibroblasts were grown to confluency in 10% FBS, and ECM was prepared on 24-well Primaria plates as described above. The ECM was rinsed twice and incubated overnight with purified IGFBP-5 (80 ng/ml) in 0.25 ml binding buffer at 4°C. Control cultures were exposed to buffer alone. The following day a separate type of human fibroblast cells (GM498; NIGMS Human Genetic Mutant Cell Repository), that had been maintained in 10% calf serum as described for GM10 fibroblasts was trypsinized and plated onto the ECM at 8,000 cells/cm$^2$ in serum-free EMEM supplemented with 0.01% BSA/linoleic acid (Sigma Chemical Co.). The GM498 cells were chosen because they secrete very low amounts of IGFBP-5 by ligand blot and immunoblot analyses of their conditioned media and ECM. The cells were allowed to attach and become quiescent overnight at 37°C. The medium was then changed and the cells incubated for 48 h at 37°C in the

absence of serum (in EMEM supplemented with 0.01% BSA/linoleic acid) in the presence or absence in the medium of IGF-I 20 ng/ml, IGFBP-5 80 ng/ml, or IGF-I plus IGFBP-5. At that time cell number was determined in a particle data counter (model ZBI; Coulter Electronics, Hialeah, FL). The ECM was removed from duplicate plates treated identically, and was analyzed by immunoblot analysis to determine the amount of IGFBP-5 present in the ECM during the growth period.

Analysis of the amount of IGFBP-5 remaining in the ECM at the end of the 48-h growth period showed that it had diffused out of the ECM substratum when the growth medium had not been supplemented with IGFBP-5. Since we had determined that IGFBP-5 would bind to type IV collagen (see Fig. 5), we therefore also performed growth studies using collagen as a substratum in order to obtain a more stable concentration of IGFBP-5 in the matrix. Tissue culture wells were coated with 20 $\mu$g of type IV collagen (Sigma Immunochemicals) and incubated overnight with 1 $\mu$g/ml recombinant human IGFBP-5 or buffer alone. Fibroblasts (GM498) were plated the next morning, and the 48-h growth study performed as described above, except that platelet-poor human plasma (0.2%) was added to the media to support attachment and growth on collagen. Platelet-poor plasma was not required for attachment and growth on ECM. Ligand blot analysis of the collagen substratum was performed at the end of the growth period, and a laser scanning densitometer (Hoefer Scientific, San Francisco, CA) was used to quantitate IGFBP-5 band intensities.

## Results

### Ligand Blot and Immunoblot Analysis

IGF binding proteins were detected by ligand blots (probed with $^{125}$I-IGF-I) in both the culture medium (Fig. 1, lanes 1 and 2) and in the extracellular matrix (lanes 5 and 6) of cultured fetal fibroblasts. The conditioned medium (lanes 1 and 2) contained bands of intense IGF-I binding activity of 39,000–43,000 $M_r$ as well as a less intense band of 24,000 $M_r$. Immunoblot analysis has previously shown that the 39,000–43,000-$M_r$ forms are IGFBP-3 and that the 24,000 form is IGFBP-4 (7). Cells cultured in calf serum (lane 2) secreted slightly more IGFBP of all species into the medium than did cells cultured in FBS (lane 1). In the ECM ligand blots (lanes 5 and 6) a 31,000-$M_r$ band was present in addition to IGFBP-3. Immunoblot analysis of the ECM (lanes 7 and 8) demonstrated that the 31,000-$M_r$ IGFBP in ECM was immunoreactive IGFBP-5. Immunoblot analysis of the conditioned medium for IGFBP-5 (lanes 3 and 4) did not detect a 31,000-$M_r$ IGFBP, but only detected a lower molecular weight band of 22,000 $M_r$. This smaller form did not bind IGF-I (lanes 1 and 2), was not detected in the ECM (lanes 7 and 8), and was shown to be a proteolytic fragment of IGFBP-5 by amino acid sequencing (7). The ECM from cells cultured in FBS (lanes 6 and 8) contained substantially less IGFBP-5 than the ECM from cells cultured in calf serum (lanes 5 and 7). Because of this observation we used ECM from cells cultured in FBS in subsequent experiments characterizing the effects of IGFBP-5 added exogenously to ECM, in which it was important to minimize the amount of endogenous IGFBP-5. The most striking difference between the ECM and the medium of these fibroblasts was that IGFBP-5 was present intact in the ECM in amounts comparable to IGFBP-3, while IGFBP-3 was the dominant IGF binding protein in the conditioned medium, in which IGFBP-5 was present chiefly as non-IGF-binding fragments. In contrast, IGFBP-5 fragments were never detected in fibroblast ECM. IGFBP-4 was present only in the medium and not in the matrix. We have not been able to detect IGFBP-2 or IGFBP-4 in ECM preparations from cell types that are able to secrete these binding proteins, while
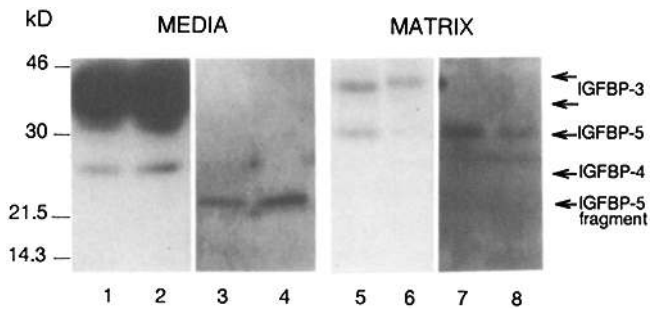
*Figure 1.* Ligand blot and immunoblot analysis of fibroblast conditioned media and ECM obtained from cells cultured in FBS or calf serum. Media (25 μl, lanes *1-4*) conditioned for 48 h and ECM (extracted from 0.5 cm² surface area, lanes *5-8*) deposited by confluent cultures of GM10 fibroblasts were subjected to SDS-PAGE in 12.5% gels. Proteins in the gels were transferred to polyvinylidene difluoride filters, and the filters were ligand blotted with 150,000 cpm/ml ¹²⁵I-IGF-I (lanes *1, 2, 5,* and *6*). The same filters were then immunoblotted with antiserum to IGFBP-5 (lanes *3, 4, 7,* and *8*). The autoradiographs demonstrate ¹²⁵I-IGF-I binding (lanes *1, 2, 5,* and *6*, exposure time 72 h) or chemiluminescence due to antibody binding (lanes *3, 4, 7, 8,* exposure time 4 min). The arrows show the locations in the blots of the IGFBP-3 doublet (in *1, 2, 5,* and *6*), intact IGFBP-5 (in *5, 6, 7,* and *8*), IGFBP-4 (in *1* and *2*), and IGFBP-5 fragments (in *3* and *4*). The IGFBP-5 fragments bind IGF-I with low affinity and therefore are not visible in lanes *1* and *2*. The samples in lanes *2, 4, 5,* and *7* were obtained from GM10 fibroblasts cultured in calf serum, while lanes *1, 3, 6,* and *8* were from the same cells cultured in FBS.

IGFBP-1 is detectable only in ECM from cells that secrete large amounts (J. I. Jones, unpublished data).

## Immunocytochemistry

Immunocytochemical staining of the fibroblast ECM preparations (Fig. 2) demonstrated positive staining for IGFBP-5 and IGFBP-3. Strongly positive staining for fibronectin and tenascin confirmed that the procedure used to prepare ECM resulted in a valid matrix preparation. Staining for IGFBP-5 (Fig. 2 *a*) was more intense than for IGFBP-3 (Fig. 2 *c*), especially when compared to their respective control slides (Fig. 2 *b* and *d*), in which the antisera had been preabsorbed with antigen excess. Staining for vitronectin, IGFBP-1, and IGFBP-2 were indistinguishable from control staining with nonimmune rabbit serum (not shown). The most intense staining was for fibronectin, tenascin, and IGFBP-5, although positive staining for decorin and laminin also occurred (not shown).

## Inhibition of ECM Association by Increasing Ionic Strength

Not only was IGFBP-5 deposited by fibroblasts into their ECM, but purified human IGFBP-5, when incubated overnight with fibroblast ECM preparations, attached to the ECM. Increasing salt concentrations markedly decreased the association and increased the release of IGFBP-5 from fibroblast ECM preparations. Fig. 3 *a*, demonstrates that
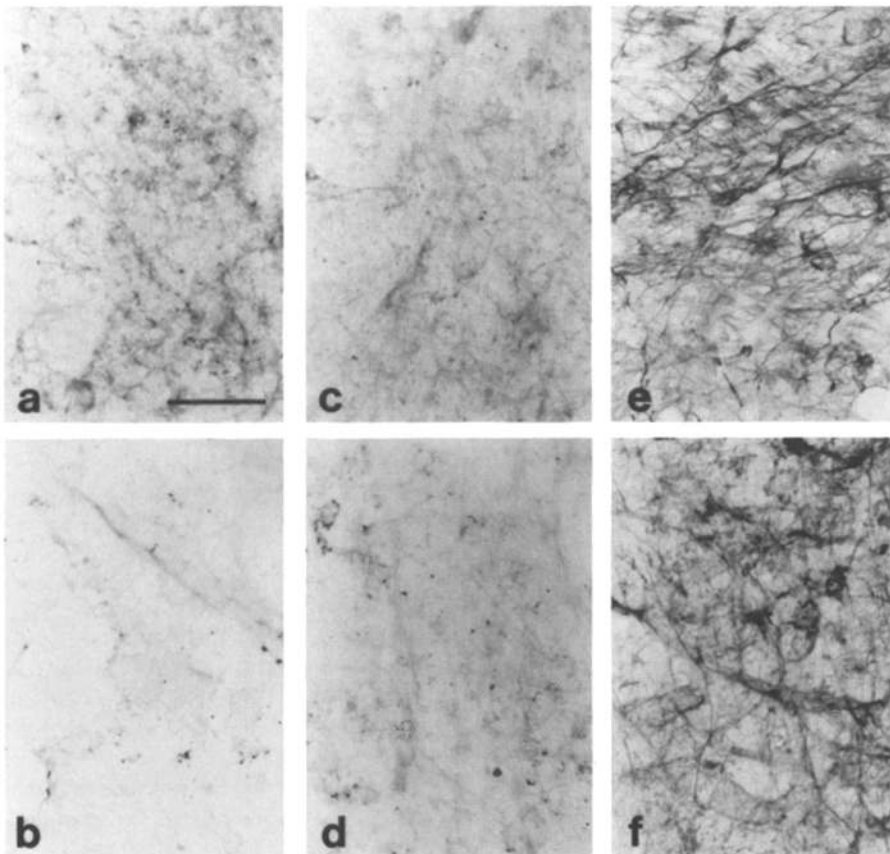


*Figure 2.* Immunocytochemistry of fibroblast ECM. ECM was prepared from confluent cultures of GM10 fibroblasts grown on glass slides. After blocking with BSA, the ECM preparations were incubated with antisera against IGFBP-5 (*a* and *b*), IGFBP-3 (*c* and *d*), fibronectin (*e*), and tenascin (*f*). To demonstrate specificity, in panels b and d the antisera were preincubated with 10 μg/ml excess pure antigen. The slides were then incubated with biotinylated monoclonal anti-IgG followed by avidin-biotin-peroxidase complex. 3,3-Diaminobenzidine was used as substrate for color development. The slides were mounted and photographed under identical exposure conditions. Bar, 50 μm.
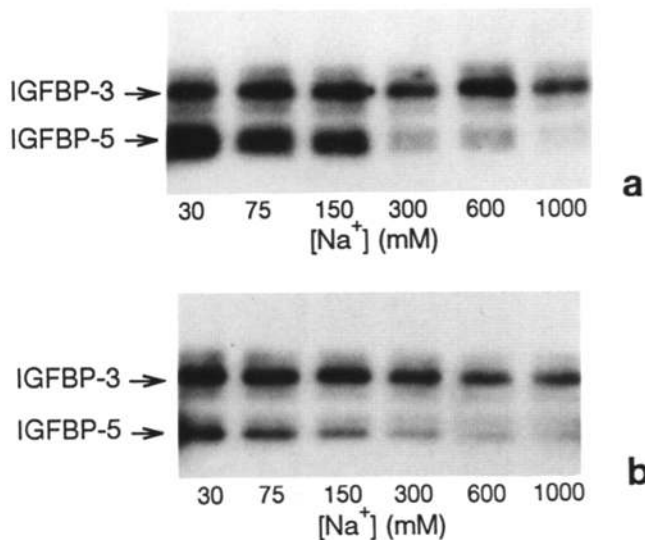
*Figure 3.* Effects of salt concentration on the attachment and release of IGFBP-5 from ECM. For the attachment experiments (*a*), fibroblasts ECM preparations were incubated with 100 ng/ml IGFBP-5 overnight in buffers containing 7, 5, 150, 300, 600, and 1,000 mM sodium and analyzed by ligand blot analysis using $^{125}$I-IGF-I. For the release experiments, ECM preparations were incubated for 5 h (*b*) at 22°C with the same buffers and analyzed by ligand blot. The arrows indicate the locations of IGFBP-5 as well as IGFBP-3 in the ligand blots.

when soluble IGFBP-5 was incubated with ECM, the greatest amount of IGFBP-5 attached to ECM and formed the most intense band in the ligand blot when the incubation was performed under conditions of minimal salt (30 mM Na⁺). There was progressive inhibition of binding with increasing Na⁺ concentrations in the buffer (from 75 mM to 1.0 M). Conversely, the endogenous IGFBP-5 deposited by the fibroblasts directly into their ECM remained associated with the ECM when incubated with a buffer containing 30 mM salt. However, increasing the Na⁺ concentration in the buffer from 75 mM to 1.0 M resulted in a progressive decrease of IGFBP-5 in the ECM which was apparent by 2.5 h and marked by 5 h (Fig. 3 *b*). While IGFBP-5 in the ECM decreased dramatically in both experiments under conditions of high Na⁺ concentration, the IGFBP-3 band intensity in Fig. 3 decreased only slightly. Immunoblot analysis of the Immobilon filters in both panels of Fig. 3 revealed that no IGFBP-5 fragment was present in the ECM at any salt concentration, and incubation of purified IGFBP-5 fragment in 30 mM sodium phosphate with ECM did not result in detectable attachment of the fragment (not shown). The concentration of IGFBP-5 in the releasate of the experiment shown in Fig. 3 *b*, was below the limit of detectability by ligand or immunoblot analyses, even after tenfold concentration. However, indirect evidence that the IGFBP-5 released from the matrix was intact rather than degraded was obtained from a quantitative IGF-I binding assay (5) performed on the releasate, which showed a progressive increase an IGF-I binding activity in releasate with increasing salt concentration, from undetectable (at 30 mM Na) to 5.3 ng/ml (at 1 M Na). IGFBP-5 fragments have an extremely low affinity for IGF-I and are not detected by this assay.
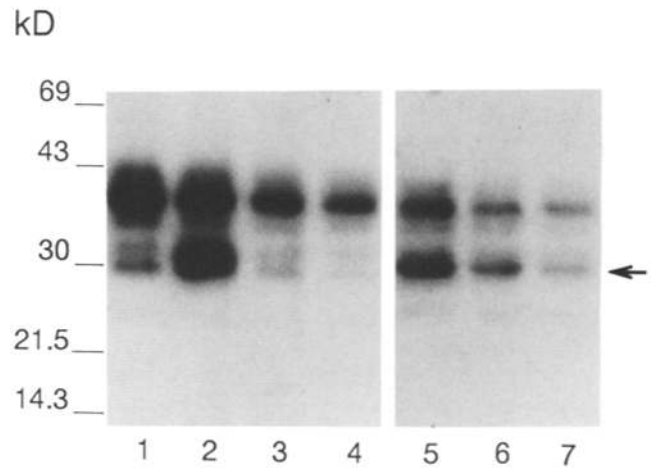


*Figure 4.* Release of exogenously added vs. endogenously synthesized IGFBP-5 from fibroblast ECM. Ligand blot analysis of ECM prepared on 96 well tissue culture wells from fibroblasts cultured in FBS (lanes *1–4*) or CS (lanes *5–7*). Lanes *1–4*: ECM immediately after preparation (lane *1*), then incubated overnight with 100 ng/ml IGFBP-5 (lane 2), then incubated in a physiologic buffer 6 h (lane *3*) or 22 h (lane 4). Lanes *5–7*: ECM immediately after preparation (lane *5*), then incubated in a physiologic buffer 6 h (lane *6*) or 22 h (lane *7*). The arrow indicates the location of IGFBP-5 in the autoradiographs.

Release of exogenous IGFBP-5 bound to ECM under physiologic salt concentration at 37°C was rapid and was complete by 6 h (Fig. 4, lane *3*). The binding and subsequent release of the purified IGFBP-5 from the ECM did not result in a loss of affinity, since a solution competition binding assay of the EGFBP-5 in the releasate determined an IGF-I binding association constant (at pH 6.0) of $6.5 \times 10^{10}$ M⁻¹ compared to $4.8 \times 10^{10}$ M⁻¹ determined for purified IGFBP-5 before binding to the ECM. Release of endogenous IGFPB-5 from fibroblast ECM was significantly less rapid under physiologic conditions, with significant amounts of IGFBP-5 remaining after 22 h (Fig. 4, lane 7).

### Binding of IGFBP-5 to Components of ECM

Purified IGFBP-5 was incubated overnight with individual matrix components immobilized on plastic microtiter wells, and the amount of IGFBP-5 which bound to each component was determined by ligand blot analysis (Fig. 5). Under these conditions, the largest amount of IGFBP-5 bound to type IV collagen, with significant binding to laminin, type III collagen, and fibronectin. The amounts of IGFBP-5 binding to types I, V, and VII collagen and to vitronectin were not greater than to control (noncoated) microtiter wells.

### Binding Affinity of ECM-associated vs. -soluble IGFBP-5 for IGF-I

After attachment to ECM or collagen substrata, IGFBP-5 retained its capacity to bind IGF-I, but its affinity was decreased (Fig. 6). Scatchard analysis of binding to IGFBP-5 using $^{125}$I-IGF-I in competition with unlabeled IGF-I demonstrated a sevenfold decreased affinity when the IGFBP-5 was immobilized on the ECM and greater than tenfold when it was immobilized on type IV collagen, compared to its
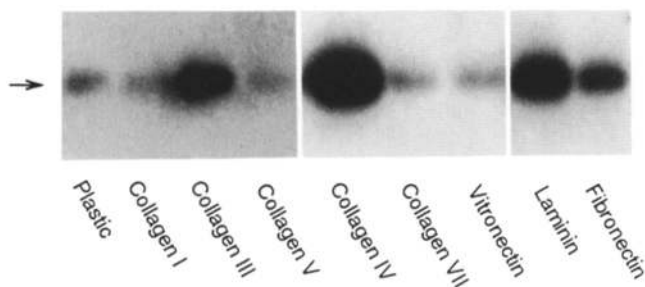
*Figure 5.* Binding of IGFBP-5 to individual ECM components. IGFBP-5 was incubated with ECM components immobilized on microtiter wells, and the attached proteins solubilized and analyzed by SDS-PAGE and ligand blot analysis. The arrow denotes the position of the IGFBP-5 band. The figure represents data from two representative experiments. Ligand blots from separate experiments were normalized by using the binding to type IV collagen as internal standards.

binding affinity when it was in solution. The x axis intercepts of the Scatchard plots indicate that the preincubation of IGFBP-5 (100 ng/ml) with the ECM- or collagen-coated plates resulted in the attachment of approximately sixfold more IGF-binding sites than were present in the IGFBP-5 (16 ng/ml) that was added directly to the solution binding assay. In contrast to IGFBP-5, relatively little IGFBP-3 attached to the matrix during an overnight incubation, resulting in too little IGF-I binding to permit Scatchard analysis (Fig. 6). This was consistent with the observation in Figs. 1 and 2 that IGFBP-5 is preferentially deposited into matrix, despite very high concentrations of IGFBP-3 in the medium.
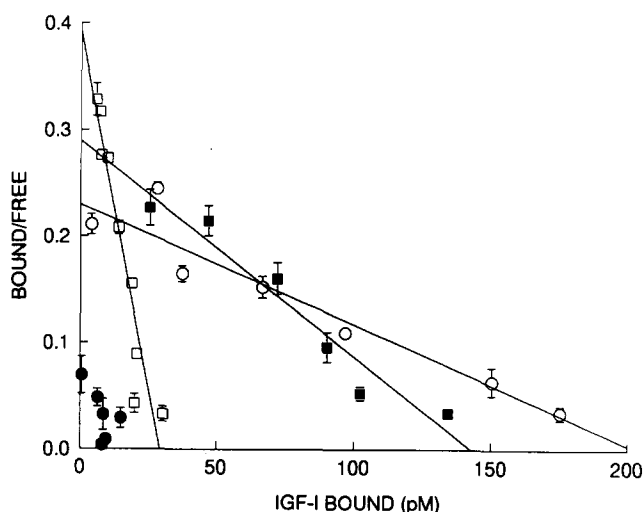


*Figure 6.* Equilibrium binding of IGF-I to IGFBP-5 in solution (□), IGFBP-5 bound either to fibroblast ECM (■) or to collagen (○), or to ECM-bound IGFBP-3 (●). $^{125}I$-IGF-I and competing concentrations of unlabeled IGF-I (0–32 ng/ml) were used as ligands. The data are plotted according to the method of Scatchard (37). Linear regression lines are shown for the IGFBP-5 data and were used to calculate association constants ($K_a$) of $1.4 \times 10^{10}$ $M^{-1}$ for soluble IGFBP-5, $2.1 \times 10^9$ $M^{-1}$ for ECM-bound IGFBP-5, and $1.1 \times 10^9$ $M^{-1}$ for collagen-bound IGFBP-5. Minimal IGFBP-3 attached to the ECM, resulting in IGF-I binding that was marginally greater than IGF-I binding to ECM alone. Error bars represent the SE of triplicate determinations.
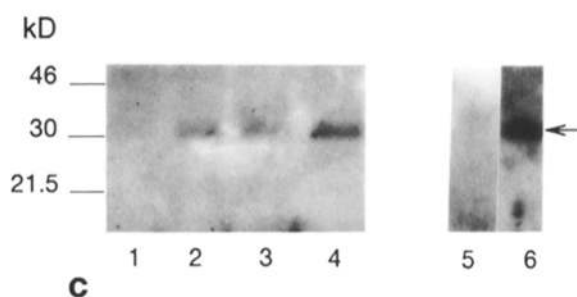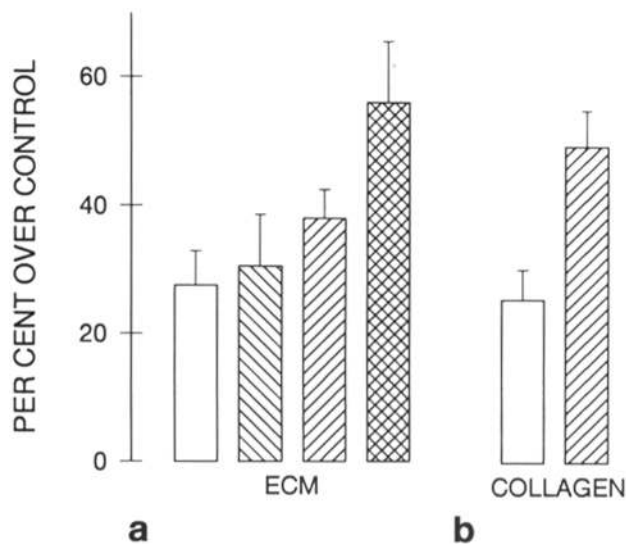


*Figure 7.* Effects of IGFBP-5 on IGF-I stimulation of fibroblast growth. Human fibroblasts were plated onto substrata consisting of fibroblast ECM (*a*) or Type IV collagen (*b*) and incubated for 48 h in serum-free EMEM (a) or EMEM + 0.2% platelet-poor plasma (*b*). Purified human IGFBP-5 was either omitted from the assay (□), preincubated with the substrata prior to plating the cells (▨), added to the medium during the 48 h growth period (▨), or preincubated with the substrata and added to the medium (▨). The height of each bar indicates the percent stimulation of proliferation achieved by addition of IGF-I (20 ng/ml) compared to the same conditions in the absence of IGF-I, for each of the four IGFBP-5 incubation conditions. The error bars indicate the SEM for triplicate determinations in three to five separate experiments. To determine maximal 48 h response, cells were also exposed to 10% calf serum, which resulted in an increase in cell number of 175% over serum-free controls. (*c*) ECM (lanes *1–4*) and Type IV collagen (lanes *5–6*) substrata were incubated with (lanes *3, 4,* and *6*) or without (lanes *1, 2,* and *5*) IGFBP-5 precisely as described for the growth assay. Fibroblasts were plated, allowed to attach, and incubated 8 h (lanes *1–4*) or 48 h (lanes *5–6*) in serum-free media containing 20 ng/ml IGF-I with (lanes 2 and *4*) or without (lanes *1, 3, 5,* and *6*) 80 ng/ml IGFBP-5. The cells were then removed with 0.5% Triton X-100 in PBS followed by 20 mM ammonium acetate, pH 9.0. The substrata were then solubilized in SDS sample buffer and analyzed by SDS-PAGE and immunoblotting for IGFBP-5. The arrow locates the IGFBP-5 bound to the substrata. Each lane corresponds to the bar directly above it in (*a*) and (*b*). The results shown in lanes 4 and 6 suggest a correlation between the amount of IGFBP-5 present in the substratum and the growth response to IGF-I.

### Potentiation of IGF-I-stimulated Cell Growth by ECM-associated IGFBP-5

To determine whether ECM-bound IGFBP-5 could alter cellular responsiveness to IGF-I, ECM was prepared and exposed to IGFBP-5, and the growth response to IGF-I determined in fibroblasts plated onto the ECM preparations in serum-free medium (Fig. 7 a). The ECM was prepared from fibroblasts cultured in FBS to minimize endogenous IGFBP-5 deposition into the matrix. In the absence of exogenous IGFBP-5, the addition of IGF-I to the medium resulted in a 27% increase in cell number after 48 h compared to cultures exposed to medium alone. If IGFBP-5 was added to the ECM before plating the cells, the IGF-I response was potentiated and cell number increased 38% compared to control cultures not treated with IGF-I. This response occurred consistently despite the observation that most of the exogenous IGFBP-5 added to the ECM was no longer detectable after 8 h of incubation (Fig. 7 c, lane 3). If IGFBP-5 was added to the medium alone, the response to IGF-I was not different than when IGFBP-5 was absent (29.6% increase in cell number over non-IGF-treated controls). However, when IGFBP-5 was added to both the medium and preincubated with the matrix, there was a substantial increase (56.2%) in final cell number compared to non-IGF-treated control cultures. The addition of IGFBP-5 to both the matrix and the medium resulted in the greatest amount of IGFBP-5 remaining in the ECM (Fig. 7 c, lane 4). IGFBP-5 added to the medium was found by immunoblot analysis to be partially degraded by 8 h and completely degraded to fragments by the end of the 48-h growth period (data not shown). No IGFBP-5 fragment could be detected in the ECM under any experimental condition (Fig. 7 c). IGFBP-5 did not increase cell growth in the absence of IGF-I (data not shown).

A problem with the system used for the growth studies was the nearly complete loss of IGFBP-5 from the ECM in cultures that did not have IGFBP-5 added to the medium. When the ECM from such cultures was analyzed as early as 8 h into the growth period, it contained minimal detectable IGFBP-5 (Fig. 7 c, lane 3). To obtain IGFBP-5 concentrations in the substrata throughout the growth period that more closely approximated the amount of IGFBP-5 in the matrix shown in Figs. 1 and 2, culture dishes were coated first with 20 $\mu$g type IV collagen and then with either 1 $\mu$g/ml IGFBP-5 or buffer before plating the fibroblasts. These conditions resulted in a more stable reservoir of IGFBP-5 in the substratum throughout the growth period, and substantial IGFBP-5 still remained attached to the collagen after 48 h (Fig. 7 c, lane 6). As shown in Fig. 7 b, the addition of this quantity of IGFBP-5 to the substratum resulted in marked potentiation of the cellular response to IGF-I (49% increase) compared to cultures that were growing on collagen that had not been incubated with IGFBP-5 (26% increase). Extraction of the collagen matrix at the termination of the experiment and analysis by ligand blotting and scanning densitometry showed that ~25% of the IGFBP-5 that had originally attached to the collagen still remained after 48 h.

### Discussion

These studies demonstrate that human IGFBP-5 associates with the subcellular ECM of fetal, dermal fibroblasts prefer-

entially compared to other forms of IGF binding protein, suggesting that the ECM-bound IGFBP-5 may have a specialized role in localizing IGF to ECM and in mediating its actions in connective tissue. ECM-associated IGFBP-5 has a decreased affinity for IGF-I. Previous studies have shown that IGFBP-1 and IGFBP-3 have decreased IGF-I binding affinity when associated with cell surfaces (26). However, this is the first demonstration of an affinity change of an IGFBP due to binding to ECM. This affinity change is not due to denaturation of the protein, since the affinity of IGFBP-5 released from the ECM is indistinguishable from purified soluble IGFBP-5.

The source of the IGFBP-5 detected in these studies appears to be predominantly the cells. Our laboratory has previously shown that these cells contain IGFBP-5 mRNA and secrete IGFBP-5 protein (7). Our IGFBP-5 antibody, which cross reacts well with bovine IGFBP-5, detects extremely small concentrations of bovine IGFBP-5 in CS and FBS by immunoblot analysis (J. I. Jones, unpublished observations). In contrast, bovine serum contains high concentrations of vitronectin which also has a high affinity for ECM (31). Since bovine vitronectin was undetectable in our ECM preparations by immunocytochemistry, it is extremely unlikely that the much lower concentration of IGFBP-5 in serum could result in a much higher IGFBP-5 concentration in the ECM compared to vitronectin.

The effect of high salt concentration on the association of IGFBP-5 with ECM suggests that an ionic interaction between IGFBP-5 and ECM components is involved. Ionic interactions are common among ECM macromolecules, particularly glycosaminoglycans, and the binding of bFGF to heparan-sulfate proteoglycans (HSPG) is inhibited by increasing salt concentrations (28). Alternatively, there may be intramolecular ionic interactions within ECM molecules which are affected by increasing ionic strength (40), resulting in unfolding or other conformational changes that affect their affinity for IGFBP-5. Analysis of IGFBP-5 affinity for individual ECM components at varying salt concentrations will further elucidate the mechanism of the IGFBP-5 interaction with the ECM. This study demonstrates that IGFBP-5 binds to types III and IV collagen as well as to laminin and fibronectin. While the kinetics and the equilibrium parameters governing the association of IGFBP-5 with individual ECM components have not been determined, the amount of IGFBP-5 that bound to type IV collagen and laminin under our experimental conditions suggests preferential binding of IGFBP-5 to components of basement membranes. In support of this hypothesis is the observation that IGFBP-5 binds in large amounts to an artificial basement membrane gel (A. Gockerman, unpublished observations). In the fibroblast ECM, the immunocytochemistry and the binding data suggest that IGFBP-5 is mostly bound to fibronectin and type III collagen (and possibly to tenascin).

The change in affinity of IGFBP-5 may have physiologic importance. In vivo, the lower affinity of ECM-bound IGFBP-5 could facilitate delivery of IGF to cell surface IGF type I receptors from reservoirs of immobilized IGF bound to low affinity IGFBP-5 in the ECM microenvironment. Furthermore, the higher affinity of IGFBP-5 in solution would allow IGF to remain bound during intercellular transport. If by being bound to IGFBP-5, IGF is protected from degradation (as is the case for, e.g., bFGF bound to HSPG; 36)

and/or inactivated (as is the case for, e.g., TGF-$\beta$ bound to decorin (44), then the high affinity of IGFBP-5 for IGF during transport followed by a lowering of its affinity after matrix localization near IGF cell receptors would be particularly advantageous. The potential importance of the affinity shift is supported by the observation that low affinity dephosphorylated IGFBP-1 (20) potentiates the effects of IGF-I on fibroblast growth (11), while high affinity phosphorylated IGFBP-1 does not (5). Likewise, an IGFBP-3 fragment with lower affinity than intact IGFBP-3 for IGF-I potentiates IGF-I effects on osteoblasts while intact IGFBP-3 is inhibitory (38). These observations are consistent with our finding in this study that when IGFBP-5 is bound to either ECM or type IV collagen, it has both a lower affinity for IGF-I and a greater capacity to potentiate IGF-I than it does when it is in solution. It may be a general property of IGF binding proteins that they inhibit IGF actions when in high affinity states ($K_a \approx 10^{10}$ M$^{-1}$) and potentiate IGF actions when their affinity is lower and approximates the affinity of the IGF-I receptor ($K_a \approx 10^9$ M$^{-1}$).

We observed an increase in IGF-I–mediated cell proliferation when fibroblasts were incubated with ECM or collagen containing added IGFBP-5. The ability of IGFBP-5 to potentiate IGF-I–stimulated growth correlated well with the amount of IGFBP-5 in the ECM during the growth period. The apparent synergistic effect of IGFBP-5 added to the medium as well as to the ECM appeared to result from an enhancement of the amount of IGFBP-5 remaining in the matrix rather than a direct effect of soluble IGFBP-5, since IGFBP-5 added to the medium alone had no effect on the IGF-I potentiation of cell growth. Since under our experimental conditions, exogenous IGFBP-5, when added only to the matrix, was in large part lost from ECM, the effects we observed likely underestimate the effects that would result from a stable pool of matrix-bound IGFBP-5 such as might be present in connective tissue. The prediction that in the tissues the IGFBP-5 pool is more stably associated with the matrix than in cell culture is supported by our observation that endogenous IGFBP-5 incorporated into the ECM by the fibroblasts disappears from ECM more slowly than does IGFBP-5 added exogenously (Fig. 4). Increasing the amount of IGFBP-5 present in the substratum by using collagen-coated plates resulted in enhanced potentiation of the IGF-I response. IGFBP-5 added directly to the medium is degraded to fragments. In contrast, the IGFBP-5 is present in the ECM only in an intact form. Whether the matrix-bound IGFBP-5 is protected from proteolysis or is released from the matrix as a result of proteolysis was not determined by these studies, but either process would result in predominantly intact IGFBP-5 being present in the matrix.

Our results suggest that the matrix-associated IGFBP-5 has physiologic significance in mediating the mitogenic effects of IGF-I. We have observed no direct effect of IGFBP-5 on cell proliferation in the absence of IGF-I. The mechanism by which IGFBP-5 potentiates IGF-I is unknown, but the binding protein likely serves to stabilize the concentration of IGF-I in the microenvironment in the vicinity of the IGF-I cell receptors. Soluble IGFBP-5 would be expected to demonstrate less potentiation of IGF-I effects for two reasons (in addition to the susceptibility of soluble IGFBP-5 to degradation). One, the soluble IGF–IGFBP complex while not being anchored in the ECM could freely diffuse away from the receptors, and two, the high affinity of the soluble IGFBP-5 for IGF would drive the IGF equilibrium away from the receptor toward the binding protein in solution. Both effects would result in fewer interactions between IGF and its cell receptors and therefore less biologic effect.

Investigation of the interactions between growth factors and the ECM has been expanding rapidly in recent year (29). Basic FGF is present in ECM (42) and associates with HSPGs both in the matrix as well as on cell surfaces (21). HSPGs serve as a low affinity/high abundance reservoir of bFGF and concentrate the growth factor in the pericellular space in the vicinity of the receptors (2, 33). The HSPGs also serve to protect bFGF from proteolytic cleavage, extending the half-life of bFGF while bound in the ECM (36). Perhaps most significantly, binding to heparan sulfate or HSPG not only potentiates the biologic activity of bFGF, but is required for bFGF to be able to bind to the cell surface receptors (22, 32, 45). An analogous interaction may occur among IGFBP-5, IGF-I, and the IGF-I receptor, resulting in potentiation by IGFBP-5 of the biologic effects of IGF-I. Alternatively, IGFBP-5 might interact directly with cell surface receptors.

There are other examples of molecules which do not have intrinsic signal-transducing activity but bind growth factors and modulate the interaction between growth factors and high affinity cell surface receptors. These include the proteoglycans decorin and betaglycan which serve as binding proteins for TGF-$\beta$ (1, 44), a 75-kD low affinity membrane protein which binds nerve growth factor (16), the $\alpha$ chain of the interleukin-2 receptor (15), and the interleukin-6 ligand-binding protein gp 130 (17, 41). Finally, there are also examples of the binding of growth factors other than bFGF and TGF-$\beta$ to components of ECM. These include granulocyte/macrophage colony stimulating factor and interleukin-3 (34), bone morphogenic proteins (43), and the product of the wnt-1 proto-oncogene (4). These examples suggest that in time it may be considered the rule rather than the exception that growth factors have accessory binding proteins that regulate access to cell surface receptors. Considered in this context, IGFBP-5 may serve an important role in IGF physiology. With ECM-associated IGFBP-5 serving not only to localize but also to stabilize matrix IGF concentrations and to facilitate receptor interactions, this ECM-associated IGFBP may be an important mediator of the paracrine actions of the IGFs.

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