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INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN-1 INHIBITS BINDING OF IGF-I ON FETAL SKIN FIBROBLASTS BUT STIMULATES THEIR DNA SYNTHESIS

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Insulin-like growth factor-binding protein-1 (IGFBP-1) was purified from human midtrimester amniotic fluid using monoclonal anti-IGFBP-1 affinity column. Two peaks were obtained in anion exchange chromatography. Both had the same molecular mass of 30 kDa. In monolayer cultures of fetal skin fibroblasts both forms of IGFBP-1 inhibited binding of [^{125}I]IGF-I onto the cells, but amplified the IGF-I-stimulated [^{3}H]thymidine incorporation into the same cells. Radiolabeled IGFBP-1 did not bind to the cells. No detectable IGFBP-1 was released into conditioned medium from the cells, and they contained no specific IGFBP-1 mRNA. Recently we found that the same IGFBP-1 preparation inhibits IGF-I-stimulated [^{3}H]thymidine incorporation into human hyperstimulated granulosa cells (1). These results show that, depending on target cells, the same protein is capable of either stimulating or inhibiting DNA synthesis.

Insulin-like growth factors (IGFs) bind with high affinity to their receptors and binding proteins (BPs). Several IGFBPs with molecular masses from 24 to 150 kDa have been purified and characterized from various body fluids and cell lines. In peripheral blood the major form is 150 kDa IGFBP, which has acid stable and acid labile subunits (2). The acid stable subunit, IGFBP-3, is well characterized and its cDNA has been cloned (3). Another binding protein, IGFBP-1 has been purified from amniotic fluid (4,5). Several groups have independently reported the isolation of cDNA molecules encoding IGFBP-1 (6-9). The gene resides in the short arm of chromosome 7 (10). The isolation of genomic clones containing IGFBP-1 shows that the gene is present in the human genome as a single copy, organized in four exons (11,12). Complementary DNA of yet another human binding protein, IGFBP-2, has also been cloned (13), and this protein appears to be a human equivalent of rat BRL-3A BP. IGFBP-2 has been purified from human cerebrospinal fluid (14). The primary structures of IGFBP-1, IGFBP-2 and IGFBP-3 have been deduced from their cDNA sequences. While their cystein residues are conserved, the overall amino acid homology of these proteins is not remarkable.

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The various IGFBPs have been shown to either stimulate or inhibit the effects of IGF-I in different cell cultures. The effect of IGFBP-1 on IGF-I action appears to be inhibitory in endometrium (15), cultured choriocarcinoma cells (16), rat thyroid follicular cells (17), human hyperstimulated granulosa cells (1), and chick embryo pelvic cartilage *in vitro* (18). A small mol mass IGFBP has been purified from amniotic fluid, which enhances DNA synthesis in cultures of fibroblasts and smooth muscle cells (19,20). Fibroblasts produce IGFBP-3 and also smaller mol mass IGFBPs, but none of these has been identified as IGFBP-1 (21-24).

The IGFBP purified from amniotic fluid seems to be heterogeneous as regards charge. Busby and his coworkers (20) have purified with ion-exhange chromatography two 31 kDa peaks with similar physicochemical characteristics. One peak potentiates and the other inhibits IGF-I-stimulated [^{3}H]thymidine incorporation into smooth muscle cell DNA. We purified IGFBP-1 from amniotic fluid on the basis of its immunological properties using anti-IGFBP-1 antibody. This purified IGFBP-1 migrates at 30 kDa and is also heterogeneous in charge. We now demonstrate that both isoelectric forms of IGFBP-1 inhibit the binding of IGF-I onto the cell surfaces but yet they enhance the IGF-I-stimulated [^{3}H]thymidine incorporation in serum free fetal skin fibroblast cell culture.

Materials and Methods

Cell cultures: Fetal skin fibroblast cells came from biopsies for routine chromosome analysis of four fetuses. Experiments on fibroblast cultures were done after five or more passages. The cells were cultured in 6-, 24- or 48-well tissue culture plates containing Ham's F10 medium supplemented with 10% fetal calf serum (FCS), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Gibco, Middlesex, U.K.) and 2 mM L-glutamine (Gibco) for 3-4 days to reach confluence.

Insulin-like growth factor-binding protein-1 (IGFBP-1): IGFBP-1 was purified by two different methods. The first made use of hydrophobic interaction chromatography (HIC) and ion-exchange chromatography as described earlier (5). It gave one major and another minor IGFBP-1 peak with the same molecular mass but slightly different isoelectric points. The pI of the major peak was 4.9 and of the minor peak 4.8.

IGFBP-1 was also purified by affinity chromatography using monoclonal anti-IGFBP-1-Sepharose column. Ascites fluid (9 ml) from the monoclonal antibody clone F5-3F4 was precipitated with 36% Na_2SO_4 and coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The column was washed with 50 mM tris-HCl, 0.9% NaCl, 0.05% NaN_3, pH 7.7 (TBS) and 1% bovine serum albumin (BSA). Then the column was washed with 0.1% trifluoro acetic acid containing 1 mM $CaCl_2$, and equilibrated with TBS. Amniotic fluid (25 ml, flow rate 5 ml/h) was applied to the monoclonal anti-IGFBP-1 column. Three washing steps were used: 120 ml of TBS, 100 ml of 1M NaCl containing 1% isopropanol and 85 ml of 10 mM ammonium acetate containing 0.005% isopropanol, pH 5.0. Elution was achieved with 0.1% trifluoro-acetic acid containing 1mM $CaCl_2$ (flow rate 10 ml/h). Fractions (5 ml) were collected into tubes containing 200 µl of 1 M Tris-HCl buffer, pH 9.0. The IGFBP-1 containing fractions were pooled and dialyzed against 10 mM sodium phosphate, pH 7.1. They were applied to a Mono Q column and eluted with a sodium phosphate gradient as described in the first method (5), concentrated and lyophilized.

Antibodies to IGFBP-1: Production of polyclonal antibodies against IGFBP-1 has been described elsewere (25). This antiserum was used for radioimmunoassay (RIA) and immunofluorometric assay (25,26).

Monoclonal antibody to IGFBP-1: BALB/c mice were from the Zentralinstitute für Versuchstiersucht (Hannover, FRG). They were immunized intraperitoneally with 60 µg IGFBP-1, purified by the HIC method, emulsified in Freund's complete (first injection) or incomplete adjuvant two times at three-week intervals. After nine weeks three boosters (15 µg, 50 µg and 10 µg) were given at four-day intervals. The last injection was given intravenously in saline four days before fusion. The fusion was performed according to Köhler and Milstein (27).

[^{125}I]IGF-I binding to fibroblast monolayers: After the fibroblast monolayers (24wells/plate) became confluent they were washed two times with Dulbecco's phosphate-buffered saline (DPBS). The cells were precultured with 0.5 ml Dulbecco's minimum essential medium (DMEM) containing 0.1% BSA alone or in combination with 500 ng/ml IGFBP-1 (either peak a or peak b). After overnight incubation the wells were washed three times with PBS. Indicated amounts of IGFBP-1 or IGF-I (KabiGen, Stockholm, Sweden) with [^{125}I]IGF-I (30,000 cpm/well, Amersham International, Buckinghamshire, U.K.) in 0.5 ml 0.1% BSA-DMEM were added to wells in triplicate. After 4 h incubation at 4°C the monolayers were washed three times with cold PBS and solubilized with 0.5 ml 0.5 M NaOH, and radioactivity of the cells was counted.

For affinity cross-linking the confluent cells (6 wells/plate) were preincubated with 0.1% BSA-DMEM for 24 h and washed twice with DPBS (0°C). IGFBP-1 (peak a plus peak b; 5, 20, 100 and 1000 ng/ml) or IGF-I (1 µg/ml) in 0.4 % BSA-DPBS or medium alone (control) were added to the wells together with [^{125}I]IGF-I (400,000 cpm/well) and incubated for 17 h at 4°C. The monolayers were washed and the cross-linking reaction was carried out with 0.5 mM disuccinimidyl suberate (Pierce Chemicals Co, Rockford, IL) as described earlier (16). The cell lysates were analysed in 5-15% SDS-PAGE according to Laemmli (28), and autoradiographed.

Binding of IGFBP-1 to cell surface: Affinity purified IGFBP-1 was labeled with Na^{125}I (Amersham) using the lactoperoxidase method as described before (29). After the monolayers (24 wells/plate) had reached confluence they were washed with PBS and incubated with radioactive IGFBP-1 (250,000 cpm/well) either alone or with IGFBP-1 (50 and 200 ng/ml) or IGF-I (5 and 20 ng/ml) at 37°C or 4°C for 16 h. Monolayers were washed again and solubilized with 0.5 M NaOH, and radioactivity of the cells was counted.

Thymidine incorporation: Fibroblasts were seeded into 48-well tissue culture plates at a density of 1.5 x 10^4 cells/well in Ham's F10-10% FCS and grown to confluence. The cells were washed and preincubated with 0.1% BSA-DMEM for 24 h. The medium was replaced with 200 µl medium containing IGF-I alone (1-100 ng/ml), or IGF-I (20 ng/ml) (Fig.4a) containing graded amounts of IGFBP-1 (5-1000 ng/ml), or with IGFBP-1 alone (5-1000 ng/ml). The wells were first incubated for 16 h (20) at 37°C with added peptides, [^{3}H]thymidine (Amersham) was added, 0.5 µCi/well, and incubation was continued for another 4 h. The medium was removed and 200 µl of cold 5% tricloroacetic acid (TCA) was added. After 10 min the cells were washed three times with cold 5% TCA and solubilized with 250 µl of 0.5 M NaOH. NaOH was transferred into scintillation vials containing RIA-luma (Lumac bv, Landgraaf, The Netherlands) and counted in a scintillation counter. All experiments were carried out in quadruplicates and repeated at least three times.

For measuring the endogenous IGF-I secretion from fibroblasts the cells were seeded into 6-well plates at a density of 9 x 10^4 cells/well and grown to confluence in 2 ml medium. After 24 h serum deprivation the IGF-I concentration was measured by RIA (Incstar Corp., Stillwater, MN, U.S.A.). Poly(A)-RNA was isolated from fibroblasts, and RNA blot hybridization for IGFBP-1 was performed as described earlier (6).

Results

IGFBP-1: The recovery of immunoreactive IGFBP-1 purified by monoclonal anti-IGFBP-1-affinity column was 40%. IGFBP-1 purified by this method, or by HIC (5), eluted from Mono Q column as two major immunoreactive peaks (fig 1a) as measured by IFMA using monoclonal or polyclonal antibodies. Both peaks had the same molecular mass, 30 kDa, in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, a faint band with molecular mass of 67 kDa was seen by silver staining (fig 1b). Also this band was immunoreactive in immunoblot and ligand blot analyses (not shown). Both major peaks were tested for their effects on [^{125}I]IGF-I binding and on [^{3}H]thymidine incorporation, and pooled for further experiments.

Binding of IGF-I and IGFBP-1 to fibroblast monolayers: The effect of IGFBP-1 on the binding of IGF-I to fibroblast monolayers was tested using [^{125}I]IGF-I and purified IGFBP-1(fig.2,peak a or b). When added together IGFBP-1 inhibited the binding of radioactive IGF-I to fetal skin fibroblasts (fig. 3). Lower concentrations of IGFBP-1 (0.5 and 1.95 ng/ml) did not affect the binding of radioactive IGF-I (not shown). Preincubation

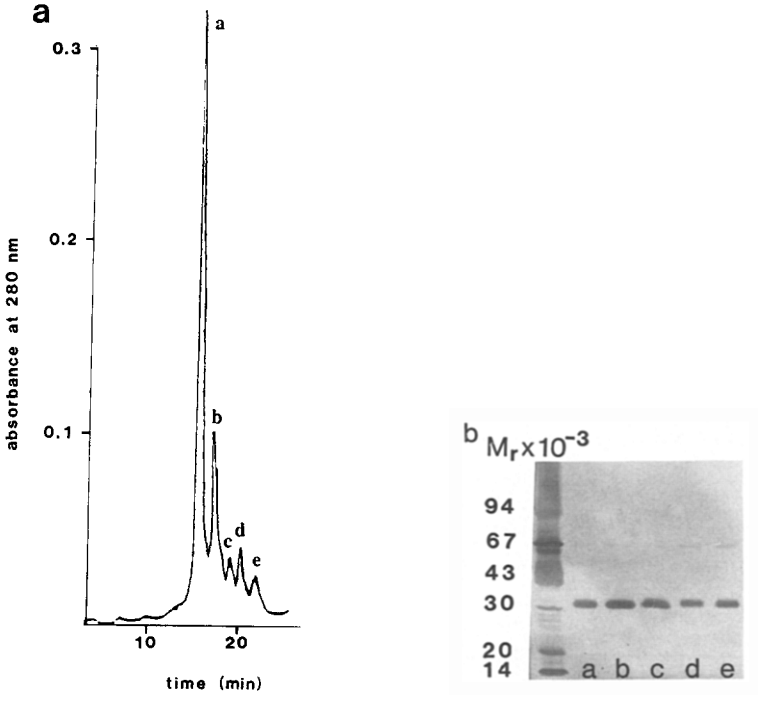
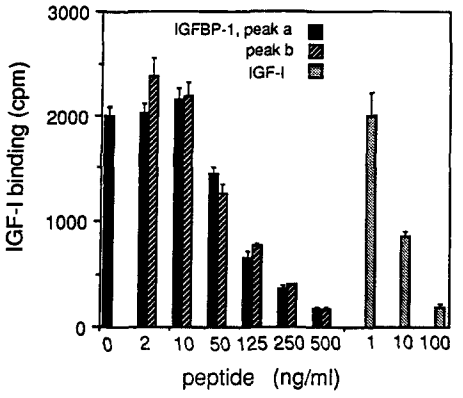
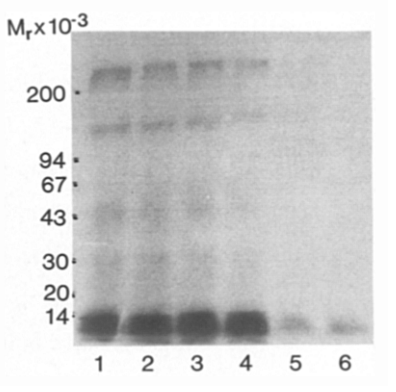


Figure 1.(a) Elution profile of affinity-purified IGFBP-1 from a Mono Q-column. The elution was achieved with increasing concentrations of sodium phosphate as described in *Materials and Methods*. (b) SDS-PAGE (12.5%, PhastSystem, Pharmacia, Uppsala, Sweden) of different IGFBP-1 peaks (a,b,c,d,e) from Mono Q-column. The gel was stained with silver nitrate.



(2)



(3)

Figure 2. The effect of IGFBP-1 on binding of [^{125}I] IGF-I to fetal skin fibroblasts. Confluent fibroblasts were preincubated with DMEM at 37°C for 24 h, then the medium was replaced with indicated concentrations of IGFBP-1, peak a or peak b, or IGF-I and [^{125}I]IGF-I. The binding reaction was carried out at 4°C for 4 h and, after washing, radioactivity of the cells was counted. At least three similar experiments were done. The data are mean ± SD for triplicate wells. IGFBP-1 significantly inhibited [^{125}I]IGF-I binding with concentrations higher than 50 ng/ml (Student's *t*-test, $P<0.001$).

Figure 3. Autoradiography of [^{125}I]IGF-I cross-linked to fetal skin fibroblasts. Monolayers were incubated with [^{125}I]IGF-I alone (line 1) or in combination with IGFBP-1 (lane 2, 5 ng/ml; lane 3, 20 ng/ml; lane 4, 100 ng/ml; lane 5, 1 μg/ml) or IGF-I (lane 6, 1 μg/ml) at 4°C for 17 h, and cross-linked with disuccinimidyl suberate as described in *Materials and Methods*.

with IGFBP-1 did not have any significant effect on the binding of [^{125}I]IGF-I to the fibroblasts.

Affinity cross-linking shows that [^{125}I]IGF-I binds to both IGF-I and IGF-II receptors as shown before (30), and also to two other proteins with molecular masses of about 24 and 35 kDa. IGFBP-1 (1 μg/ml) inhibits the binding of [^{125}I]IGF-I to these proteins. Smaller amounts of IGFBP-1 have no effect to the binding (fig. 3). No detectable binding to the cells (less than 0.2%) of radiolabeled IGFBP-1 was observed at 37°C or 4°C.

Thymidine incorporation: The effect of IGF-I and IGFBP-1 on DNA synthesis of fetal skin fibroblasts was demonstrated by [^{3}H]thymidine incorporation into the cells. IGF-I had a moderate stimulating effect on thymidine incorporation. The addition of IGFBP-1 into culture medium brought about a significant increase in IGF-I (20 ng/ml)-stimulated incorporation in four experiments using fibroblasts from two donors (fig. 4). Fibroblasts from two other donors did not respond at all. The enhancement was less pronounced with high concentrations of IGFBP-1 (1000 ng/ml). Also IGFBP-1 alone at a concentration of 20 ng/ml had a slight stimulating effect on [^{3}H]thymidine incorporation into fetal skin fibroblasts ($P< 0.05$) . The fibroblasts did not secrete detectable (less than 0.1 ng/ml) IGFBP-1 as measured by RIA or IFMA. The endogenous IGF-I concentration in the

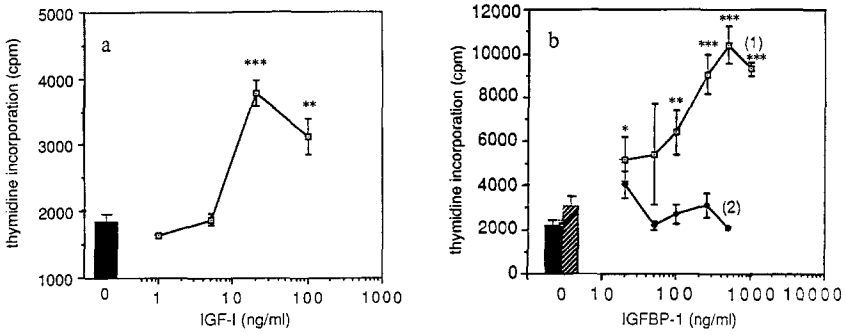


Figure 4. (**a**) IGF-I-stimulated [^{3}H]thymidine incorporation in fetal skin fibroblasts. (**b**) Enhancement of IGF-I-stimulated [^{3}H]thymidine incorporation in fetal skin fibroblasts by IGFBP-1. After 24 h serum deprivation the confluent cells were incubated (a) with IGF-I (0, 1, 5, 20, 100 ng/ml) or (b) with IGFBP-1 (0, 20, 50, 100, 250, 500, 1000 ng/ml) alone or in combination with IGF-I (20 ng/ml) at 37°C for 16 h, [^{3}H]thymidine was added to the wells and incubated for another 4 h. Curve 1, IGF-I plus IGFBP-1; curve 2, IGFBP-1 alone; *black bar*, medium alone; *hatched bar*, IGF-I alone (20 ng/ml). The results are expressed as mean ± SEM of quadruplicate determinations. (*), $P<0.05$; (**), $P<0.01$; (***), $P<0.001$.

medium was 6.5 ± 0.5 ng/ml (n=3). In RNA blot hybridization analysis the fibroblasts did not express IGFBP-1 mRNA (not shown).

Discussion

As calculated from its amino acid sequence, the molecular mass of the IGFBP-1 we used in this study is 25 kDa (6). In SDS-PAGE, nonreduced IGFBP-1 migrates at 30 kDa. We generated a monoclonal antibody against this IGFBP-1 and used it for subsequent purification of the protein from human amniotic fluid. Even this IGFBP-1 showed microheterogenity on the basis of charge. In addition to the main 30 kDa band, a faint 67 kDa band was observed in SDS-PAGE by a highly sensitive silver staining. This band was immunoreactive in immunoblot analysis using monoclonal or polyclonal antibodies. It is probably a dimer as also reported by others (20,31).

Inhibition by IGFBP-1 of binding of radioactive IGF-I onto the cells has been demonstrated to occur in other cell cultures as well (16,17), and IGFBP-1 has also been reported to inhibit the IGF-I-stimulated DNA synthesis. In our fibroblast cell cultures, IGFBP-1 inhibited binding of [^{125}I]IGF-I to the fetal skin fibroblast cells, but yet it enhanced the IGF-I mediated [^{3}H]thymidine incorporation into the same cells. The mechanisms leading to this apparently controversial observation are not clear. One possibility is that IGFBP-1 forms a complex with IGF-I which may release IGF-I to the cells at suitable small concentrations. An alternative explanation is that IGFBP-1 has an active role in transmitting IGF-I to the cells. IGFBP-3 has likewise been reported to have opposite effects depending on culture conditions (32). In human adult skin fibroblasts,

DNA synthesis is enhanced when IGF-I is complexed with IGFBP-3 before the experiment (20). Conceivably such complexes also exist *in vivo*, and the actions on various cell types of such complexes are not uniform.

Acknowledgments

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