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MONOCLONAL ANTIBODIES TO THE 27-34K INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN

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Summary: Monoclonal antibodies were prepared against the 27-34K insulin-like growth factor (IGF)-binding protein purified from human placenta/decidua and designated placental protein 12 (PP12). Four different antibodies were characterized. Each recognized the major band at 32K on immunoblots of the purified PP12 preparation and amniotic fluid. In liquid phase RIA, IGF-I did not affect the binding of [^{125}I] PP12 to one antibody (Mab 6303), it slightly increased the binding to two antibodies (Mab 6301 and 6304), and it slightly decreased the binding to one antibody (Mab 6302). All antibodies immunoprecipitated the cross-linked PP12-[^{125}I] IGF-I complex, but Mab 6302 considerably less effectively than the others. Preincubation of PP12 with Mab 6302 completely inhibited the binding of [^{125}I] IGF-I to PP12, whereas preincubation with Mab 6303 had no effect, and Mab 6301 as well as Mab 6304 increased it. These results suggest that Mab 6302 binds to an epitope at or near to the IGF-binding site, whereas the other antibodies react at other sites of the PP12 molecule. Conformational changes in PP12 probably account for the IGF-I-induced increase in the binding of Mabs 6301 and 6304 to [^{125}I] PP12, and vice versa, for Mabs 6301- and 6304-induced increase in the binding of [^{125}I] IGF-I to PP12.

Insulin-like growth factors (IGF-I and IGF-II) interact with cell surface receptors and soluble binding proteins (1,2). The physiological role of binding proteins is unclear, but present evidence suggests that they play a pivotal role in regulating the delivery of IGFs to their target cells (3-5). The main IGF-binding proteins in human serum are the 125-150K GH-dependent species and the 30-40K species (2,6), which differ in their amino-terminal sequences (7,8). The first carries most of the endogenous IGFs, whereas the latter has the majority of unoccupied IGF binding sites (6). The 30-40K species is abundant in amniotic fluid (8-11). Human IGF-binding proteins have been purified by various methods from plasma (12), amniotic fluid (8-11), placental/decidual extract (13), and culture medium conditioned by human hepatoma cells (14). Polyclonal antibodies have been raised to purified binding proteins and RIAs using these antibodies have been established by several laboratories (8-11,15).

The 34K (reduced) IGF-binding protein purified from placental/decidual extracts is designated placental protein 12 (PP12)(13). It is synthesized by human decidua, but not placenta (16,17). PP12 is identical with the 27-34K IGF-binding protein purified from human amniotic fluid (8,10,11,18). The protein binds both IGF-I and IGF-II with high affinity (11,18). In this study, we describe four monoclonal antibodies to PP12. The antibodies were also tested for their ability to recognize PP12 affinity-labeled with [^{125}I] IGF-I and to inhibit the binding of [^{125}I] IGF-I to PP12.

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MATERIALS AND METHODS

IGF-binding protein: PP12 was provided by Behringwerke AG, Marburg/Lahn, West Germany. It was purified from soluble extracts of term human placenta/decidua (13). On HPLC, purified PP12 appears as a single peak. On SDS-PAGE under reducing conditions it migrates as a major band at 32-34K and a minor band at 27-28K. Under nonreducing conditions, both bands are still present, but their migration mobilities are slightly faster, the major band being at 28-29K. The N-terminal amino acid sequence is identical with that of IGF-binding protein purified from amniotic fluid (18). Radioiodination of PP12 was carried out by the chloramine-T method. Radioiodinated PP12 also showed major and minor bands at 32-34K and 27-28K, whereas PP12 affinity-labeled with [^{125}I] IGF-I gave a diffuse band at 40K.

Polyclonal antibodies: Rabbit anti-PP12 antiserum from Behringwerke AG, was used for comparative studies. Its specificity has been previously described (13,15).

Peptides: Recombinant [Thr^{59}I]IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA). Human IGF-I was a generous gift from Prof. René E. Humbel (Zurich, Switzerland). The peptides were iodinated by the chloramine-T method and purified by gel chromatography on Sephadex G-50 at neutral pH. The specific activities of both tracers were 50-100 Ci/g.

Immunization and production of monoclonal antibodies: Six 10 to 12-week-old BALB/c mice were immunized with PP12 using the protocol described for AFP (19). All mice developed antiserum that reacted with PP12 in liquid phase RIA. Spleen cells from the immunized mice were fused with myeloma cells using polyethylene glycol (PEG), MW 1500, (J.I. Baker Chemicals, B.V.-Deventer, Holland) (20). Hybrid cells were plated in 96-well plates containing 22 x 10^5 cells/well. The fusion and hypoxanthine-aminopterin- thymidine selection procedure was carried out as described by Köhler and Milstein (20). Nine days after fusion, the supernatants were screened for antibody secretion. Antibody producing hybridomas were cloned by limiting dilution. Culture medium from established anti-PP12-secreting clones were harvested for further testing. Selected clones were grown intraperitoneally in pristane-treated mice for ascites fluid production.

Screening of the sera and hybridomas: Sera and hybridoma supernatants were assayed for anti-PP12 antibodies using liquid phase RIA, employing PEG to precipitate counts bound to antibody. Sera and supernatants were diluted to a final volume of 50 µl in RIA buffer (13 mM Na phosphate, 0.3% BSA, 33 mM EDTA, pH 7.4). [^{125}I] PP12 (10,000 cpm) in 150 µl of the same buffer was added and incubated overnight at room temperature. After PEG precipitation, the pellets were counted in a gamma counter. Nonspecific binding was subtracted from each sample value.

Purification of monoclonal antibodies: Antibodies were purified by affinity chromatography on Protein A-Sepharose according to Ey et al. (21) with slight modifications. The purity of each antibody was checked by 10-15 % SDS-PAGE. The protein concentration was adjusted to 1 mg/ml based on optical density at 280 nm.

Characterization of monoclonal antibodies: Purified antibodies were isotyped using Bio-Rad Subisotyping kit (Bio-Rad Laboratories, Richmond, CA). Liquid phase RIA was used to determine the affinity constants (K_d). The antibody solution was diluted to bind 30-40% of [^{125}I] PP12 . For the assay, 100 µl of antibody solution, 100 µl of [^{125}I] PP12, and 100 µl of standard were incubated overnight at room temperature. The bound fraction was precipitated by addition of 50 µl bovine gamma-globulin (20 mg/ml) and 450 µl 20% PEG 6000. After an additional 4 h, the pellets were counted in a gamma counter. Affinity-constants were estimated from Scatchard plots (21). To test whether the binding of these antibodies to PP12 is affected by IGF-I, both human and recombinant IGF-I at concentrations up to 2 µg/ml were incubated with [^{125}I] PP12 and each antibody.

Immunoblotting: Purified PP12 (4 µg/lane) was subjected to 2-16% SDS-PAGE under reducing conditions and transferred to nitrocellulose sheet (23). After blocking with PBS containing 3 % BSA, purified Mab or nonimmune mouse IgG diluted 1:100 into 2 % swine serum in PBS were incubated with the nitrocellulose for 1 h at 37 C. Antigen-antibody reaction vas visualized by the peroxidase-antiperoxidase method using aminoethylcarbazole (Sigma Chemical Co., St. Louis, MO). The specificity of the antibodies against PP12 in the presence of other components was analyzed performing an immunoblot from amniotic fluid.

Ability of the antibodies to immunoprecipitate the PP12-[^{125}I] IGF-I complex and to inhibit the binding of [^{125}I] IGF-I to PP12: The following methods were used to test whether the antibodies recognize the [^{125}I] IGF-I-PP12 complex and whether they inhibit its formation. 1) Purified PP12 (100 ng) was incubated with [^{125}I] IGF-I (500,000 cpm) in a total volume of 200 µl PBS, 0,1 % BSA, pH 7.4, for 16 h at 4 C. Thereafter, chemical cross-linking with disuccinimidyl suberate (DSS) (0.5 mM) was carried out as previously described (24). The cross-linked samples were immunoprecipitated with 50 µl of 1 to 10 diluted monoclonal antibodies or nonimmune mouse IgG (5 µg). Control tubes, in which PP12 was omitted, were included. After 8-h incubation, 20 µl rabbit anti-mouse serum was added. After an additional 2 h at 4 C, immunocomplexes were precipitated by Pansorbin (150 µl), washed twice with 50 mM Tris-HCl, 0.5% Triton X-100 and once with SDS-gel buffer, pH 6.8, before analysis on 10% SDS-PAGE followed by autoradiography. 2) PP12 was preincubated with antibodies (50 nM) or nonimmune mouse IgG (50 nM) for 8 h at 4 C. Thereafter, [^{125}I] IGF-I (1,000 000 cpm) was added and incubation was continued for 12 h before chemical cross-linking with DSS was carried out as above. The cross-linked PP12-[^{125}I] IGF-I complexes were analyzed by SDS-PAGE and autoradiography. 3) Receptor binding of [^{125}I] IGF-I to choriocarcinoma cells (JEG-3; American Type Culture Collection, Rockville, MD), which is inhibited by PP12 (50% inhibition at a concentration of 20 ng/ml) (25), was measured in the absence and presence of PP12 as well as in the presence of PP12 preincubated with monoclonal antibodies or nonimmune mouse IgG. PP12 (5 ng) was preincubated without and with monoclonal antibodies (50 nM) or nonimmune mouse IgG in a total volume of 250 µl of 0.1%BSA in PBS, pH 7.4, at 4C for 16 h. Thereafter, the incubation mixture was added with [^{125}I] IGF-I (30,000 cpm) to cell monolayers. Control wells containing Mabs or nonimmune mouse IgG without PP12 were included. After a 16-h incubation at 4C, the cell bound radioactivity was measured as previously described (25). In a separate experiment, 100 ng PP12 (amount that totally inhibits receptor binding of [^{125}I] IGF-I) were preincubated with various concentrations (0-50 nM) of Mab 6302 in a final volume of 0.5 ml binding buffer before added to cell monolayers with [^{125}I] IGF-I (100,000 cpm). The cell bound radioactivity was measured as above.

RESULTS

Of a total of 3178 hybrid cultures, 196 hybridomas secreting antibodies to PP12 were established. The reaction patterns of the antibodies with purified PP12 in RIA were employed for the selection of clones. Eleven subclones were developed. The affinities (K_d) of the monoclonal antibodies secreted by these clones ranged from 10^{-9} to $10^{-10}M^{-1}$. Four antibodies, selected on the basis of immunoblotting and designated Mab 6301, 6302, 6303, and 6304, were purified and further characterized. They all were of the IgG_1 subclass. In liquid phase RIA, human and recombinant IGF-I at concentrations up to 2 µg/ml had no effect on the binding of one antibody (Mab 6303), but they increased the binding of two antibodies (Mab 6301 and 6304) and slightly inhibited the binding of one antibody (Mab 6302) to [^{125}I] PP12 (Fig. 1).

Immunoblot analysis after SDS-PAGE of purified PP12 showed strong reaction of all these antibodies with a band at 32K. All exept one (Mab 6301) also showed reaction with the 27K band and with one or two additional bands with faster migration mobilities (Fig. 2). When amniotic fluid was analyzed, only a single band at 32K was seen on immunoblots with each antibody (Fig. 3).

All four antibodies immunoprecipitated the cross-linked [^{125}I] IGF-I-PP12 complex, Mab 6302 being considerable less effective than the other antibodies (Fig. 4). If PP12 was omitted from the incubation mixture, or control IgG was used for immunoprecipitation, no cross-linked bands were

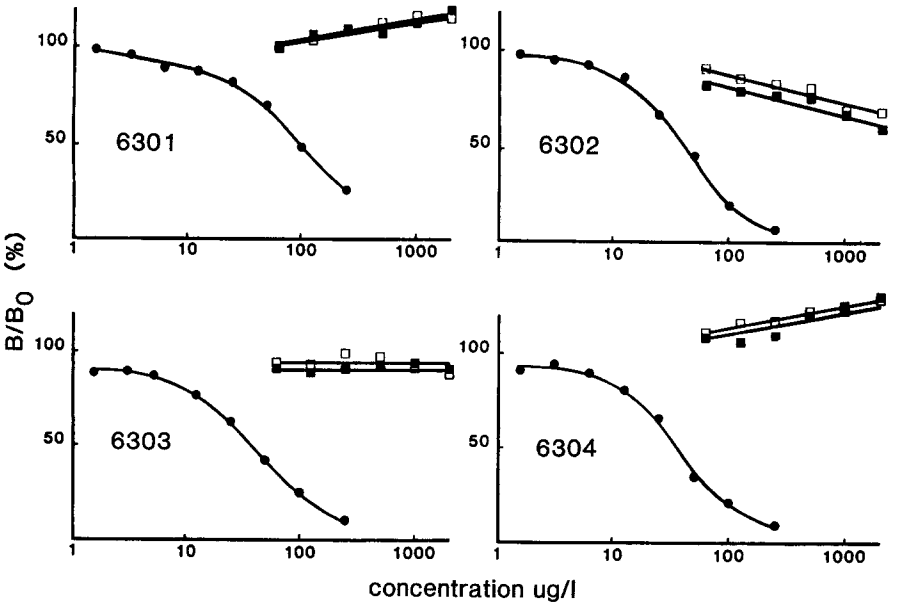

Fig. 1. Inhibition of [^{125}I] PP12 binding to four monoclonal antibodies by unlabeled PP12 (●), human IGF-I (■), and recombinant IGF-I (□) in liquid phase RIA.

visualized. The formation of radiolabeled bands was also prevented by the addition of excess unlabeled IGF-I (recombinant and human).

Mab 6302 prevented, whereas Mab 6301, 6303 and 6304 did not prevent, the binding of [^{125}I] IGF-I to PP12, as tested by affinity cross-linking. When [^{125}I] IGF-I was incubated with PP12 in the presence of Mab 6302, followed by cross-linking with DSS and SDS-PAGE, no specifically

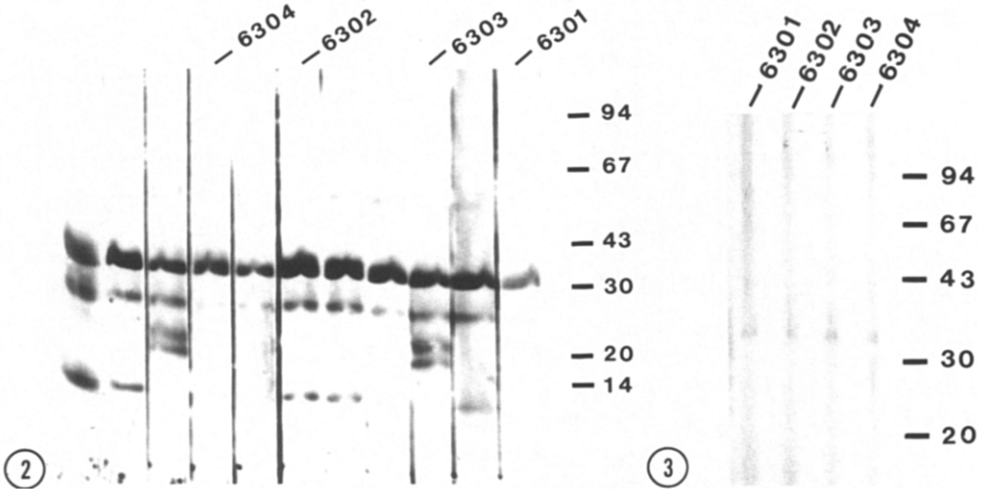

Fig. 2. Immunoblot analysis of the purified PP12 preparation with eleven different monoclonal antibodies (Mab). Mabs described in this report are marked. PP12 (4 µg/lane) was subjected to 2-16% SDS-PAGE under reducing conditions and transferred to nitrocellulose sheet. Mabs diluted 1:100 were incubated with the nitrocellulose, and the blots were visualized as described in Materials and Methods. The relative mol mass of standard proteins is indicated.

Fig. 3. Immunoblot of amniotic fluid after 10-15% SDS-PAGE under reducing conditions (Phast System, Pharmacia).

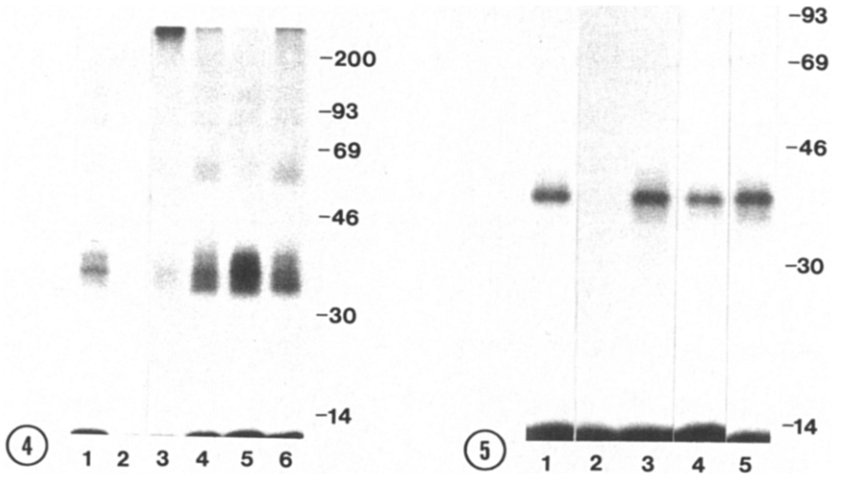

Fig. 4. Immunoprecipitation of covalently cross-linked [^{125}I] IGF-I-PP12 complex. PP12 was incubated with [^{125}I] IGF-I (500,000 cpm), followed by cross-linking and immunoprecipitation with the polyclonal anti-PP12 antiserum (lane 1), or nonimmune mouse IgG (lane 2), or Mabs (lane 3, 6302; lane 4, 6301; lane 5, 6303; lane 6, 6304). When PP12 was omitted, or excess amount of unlabeled IGF-I was present, no radiolabeled bands were visualized (not shown).

Fig. 5. Effect of the monoclonal antibodies on the binding of [^{125}I[IGF-I to PP12 as determined by affinity cross-linking. PP12 was preincubated with Mabs or nonimmune mouse IgG for 8 h, before [^{125}I] IGF-I was added. After an additional 16 h at 4 C, cross-linking with DSS was performed. The cross-linked [^{125}I] IGF-I-PP12 complexes were analyzed by 10% SDS-PAGE and autoradiography. Lane 1, 6301; lane 2, 6302; lane 3, 6303; lane 4, 6304; lane 5, nonimmune mouse IgG.

labeled bands were visualized on autoradiography. In contrast, when [^{125}I] IGF-I was incubated with PP12 in the presence of Mab 6301, 6303, or 6304, radiolabeled bands were visualized which co-migrated with bands obtained by cross-linking PP12 and [^{125}I] IGF-I in the absence of Mabs or in the presence of nonimmune mouse IgG (Fig. 5).

TABLE I

Effect of monoclonal antibodies on PP12-induced inhibition of [^{125}I] IGF-I binding to JEG-3 cells

Treatment of cells	[^{125}I] IGF-I bound (cpm)
[^{125}I] IGF-I (specific binding)	2321
plus PP12	940
plus PP12 plus Mab 6301	396
plus PP12 plus Mab 6302	2306
plus PP12 plus Mab 6303	945
plus PP12 plus Mab 6304	362
plus PP12 plus nonimmune mouse IgG	847

Specific binding of [^{125}I] IGF-I to JEG-3 cell monolayers was measured in the absence and presence of PP12, as well as in the presence of PP12 preincubated with Mabs or nonimmune mouse IgG. Nonimmune mouse IgG or Mabs alone had no effect on the binding of [^{125}I] IGF-I to JEG-3 cells (not shown). The results are mean of duplicates in a representative experiment. Similar results were obtained by human and recombinant IGF-I in three experiments.

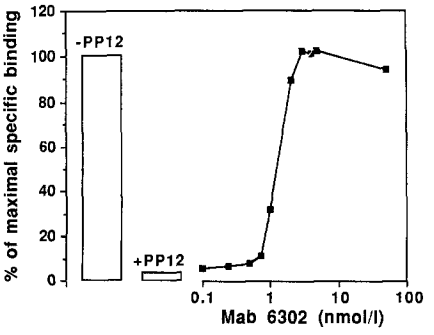

Fig. 6. Blocking of PP12-induced inhibition of the receptor binding of [^{125}I] IGF-I by Mab 6302. Increasing amounts (0-50 nM) of monoclonal antibody 6302 were preincubated with 100 ng PP12 for 8 h at 4 C in the receptor binding buffer. Thereafter, the buffer plus [^{125}I] IGF-I (100,000 cpm) were added onto the cell monolayers and receptor binding was measured as previously described (25). The results are presented as percentage of maximal specific binding of [^{125}I] IGF-I.

Specific binding of [^{125}I] IGF-I to JEG-3 cells is inhibited by PP12 (25). This inhibition is due to the interaction of PP12 with the tracer. Preincubation of PP12 with Mab 6303, or nonimmune mouse IgG, did not alter its inhibitory effect. Mab 6302 completely blocked PP12- induced inhibition of the [^{125}I] IGF-I receptor binding to JEG-3 cells at final concentrations greater than 3 nM (Table 1, Fig.6), indicating that this antibody prevented the interaction of PP12 with [^{125}I] IGF-I also in this experimental system. Monoclonal antibodies alone had no effect on the receptor binding of [^{125}I] IGF-I, indicating that they interacted neither with the receptor nor with IGF-I itself. When PP12 was preincubated with Mabs 6301 and 6304, its inhibitory effect on the receptor binding of [^{125}I] IGF-I was strengthened. Thus, these antibodies appeared to enhance the binding of [^{125}I] IGF-I to PP12. Similar results were obtained by using human and recombinant IGF-I.

DISCUSSION

In this study we describe four monoclonal antibodies against the 27-34K IGF binding protein PP12. In addition to the major 32-34K band and the minor 27-28K band, which have previously been recognized by polyclonal antiserum and Coomassie blue staining, 22K, 20K and 15K bands were identified on immunoblot analysis of the purified PP12 preparation using monoclonal antibodies. These bands presumably represent breakdown fragments of PP12, similar as has been described for the other human IGF-binding protein purified by Martin and Baxter (11). The fragments could not be visualized by cross-linking experiments, indicating that they had not retained IGF-binding capability, or that their concentrations were to low to be recognized by this technique. All antibodies showed a single band at 32K on immunoblots of amniotic fluid, indicating that they can specifically bind this IGF-binding protein species also in biological material. One of the four antibodies was unaffected by IGF-I, whereas the binding of two antibodies to PP12 was increased, and the binding of one antibody was inhibited in the presence of IGF-I. The results obtained by RIA and by immunoprecipitation of [^{125}I] IGF-I-PP12 complexes were in agreement, and support the conclusion that IGF-I and Mab 6302 compete for the same epitope on PP12. Interestingly, the same antibodies which bound more [^{125}I] PP12 in the presence

of IGF-I appeared to increase the binding of [^{125}I] IGF-I to PP12, as shown by receptor binding experiments. Mab 6303, which was unaffected by IGF-I, had no effect on the interaction of [^{125}I] IGF-I with PP12. Mab 6302, which was considerably less effective (the band almost invisible on autoradiography) than the other antibodies in immunoprecipitating the cross-linked [^{125}I] IGF-I-PP12 complex, completely inhibited the binding of [^{125}I] IGF-I to PP12, as shown by cross-linking and receptor binding experiments. These results suggest that Mab 6302 binds to an epitope at or near the IGF-I binding site, whereas the other antibodies react at other sites of PP12. The reason, why Mabs 6301 and 6304 increase the [^{125}I] IGF-I binding to PP12 and why IGF-I increases the binding of Mabs 6301 and 6304 to [^{125}I] PP12, remains unclear. Conformational alterations in PP12 provide the most likely explanation. The monoclonal antibodies described here provide new tools for studies on the structure of the 27-34K IGF-binding protein and its interaction with IGFs. In addition, these antibodies will be useful in developing assays to measure the 27-34K IGF-binding protein in its free and complex forms (under preparation).

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