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An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I

(fibroblasts/receptors/somatomedin-C/replication/growth)

R. G. ELGIN, W. H. BUSBY, JR., AND D. R. CLEMMONS*

Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, NC 27514

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ABSTRACT **The insulin-like growth factors IGF-I and IGF-II circulate in blood bound to carrier proteins. The higher molecular mass IGF-binding protein complex (150 kDa) is composed of subunits, and one subunit that forms this complex is growth hormone dependent. In addition, many cell types and tissues secrete another form of IGF binding protein that is not growth hormone dependent. Both forms of the IGF binding protein are believed to inactivate the IGFs and to function as delivery systems to tissues. This conclusion was based on studies that determined the effects of impure preparations of these binding proteins or that examined the effect of these proteins only on the insulin-like actions of the IGFs. We report here that a pure preparation of the extracellular form of the IGF binding protein (purified from human amniotic fluid) markedly potentiated replication of several cell types in response to human IGF-I. Secondary cultures of human, mouse, and chicken embryo fibroblasts as well as porcine aortic smooth muscle cells showed marked enhancement of their DNA synthesis response (2.8- to 4.4-fold increases) to IGF-I in the presence of this protein. These responses were synergistic since the sum of the responses to either IGF-I or to the binding protein alone was between 8 and 17% of the increase obtained in cultures exposed to both peptides. The binding protein not only potentiated the DNA synthesis response but also enhanced the increase in cell number in response to IGF-I. This stimulation is specific for growth factors that bind to the binding protein since incubation with insulin, which binds to the type I IGF receptor but not to the binding protein, did not result in potentiation of this response. We conclude that a form of IGF binding protein that is present in extracellular fluids and is secreted by many types of cells can markedly potentiate the cellular response to IGF-I.**

Human insulin-like growth factor I (IGF-I), also termed somatomedin C, is a growth hormone dependent growth factor that circulates in blood (1) and is synthesized in many tissues (2). Like other growth factors, such as epidermal growth factor, that circulate in plasma, the IGFs are bound to a binding protein that forms a 150-kDa carrier protein–IGF-I complex and is believed to serve a transport function. Partially purified forms of this protein have been shown to inhibit the insulin-like actions of IGF-I (3). An acid-stable binding subunit of this complex has been purified from blood, and its secretion can be stimulated by growth hormone (4, 5). In contrast, extracellular fluids such as spinal (6), lymph (7), and amniotic (8) as well as tissue extracts from brain (9) and pituitary (9) contain a form of IGF binding protein that has a different molecular mass and is not growth hormone dependent. It binds both IGF-I and IGF-II with affinities in the 10^{10}–10^{9} M^{-1} range. This protein has been shown to be secreted by some cell types in culture including human fibroblasts (10). This binding protein has been purified to homogeneity from human amniotic fluid and has molecular mass estimates between 32 and 38 kDa (8, 11). Impure preparations of this protein, or a similar protein that is secreted by rat liver cells, have been shown to inhibit the effects of IGF-I and IGF-II on fibroblast DNA synthesis (12) or on sulfate incorporation into cartilage (8), presumably by preventing the binding of IGF-I to its cell surface receptor. However, recent studies have shown that if this protein is adherent to cell surfaces it causes an increase in the amount of IGF-I that binds to the IGF receptor and to cell surfaces (10). These studies were undertaken, therefore, to determine the effect of a pure preparation of the IGF-I binding protein on IGF-I stimulated DNA synthesis in several types of cells in culture.

MATERIALS AND METHODS

Cell Culture Techniques. Porcine aortic smooth muscle cells were isolated by a previously described method (13). Stock cultures were plated at 8000 cells per cm^2 in 10-cm plastic dishes (Falcon Labware Division, Becton Dickinson, Oxnard, CA; 3001). They were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) that was supplemented with 10% fetal bovine serum (FBS) (HyClone, Ogden, UT). They were passaged every 10–12 days by removing the cells with 0.03% trypsin/0.02% EDTA (GIBCO) and replating at a 1:5 dilution. All experiments were conducted using cells between the fourth and seventh passages. Individual experiments were conducted using cultures that had been plated at 8000 cells per well in 96-well microtest plates (Falcon 3004) in 0.2 ml of DMEM supplemented with 10% FBS and had been grown for 5 days. At that time the serum-containing medium was removed, and test substances were added to 0.2 ml of DMEM supplemented with 1% human platelet poor plasma (PPP) and 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (15 Ci/mmol) (Schwartz/Mann). Control wells received DMEM containing either 1% PPP or various concentrations of human serum (1–10%). The PPP and serum were prepared using a previously described method (13). The PPP was determined by RIA (13) to contain <20 pg of platelet factor 4 per ml. Following a 36-hr incubation, the reaction was stopped and the amount of [^{3}H]thymidine that had been incorporated into DNA was determined (13).

Chicken embryo fibroblasts, which were a gift of John Olsen (Univ. of North Carolina), were isolated from 14–16 day chicken embryo skin. The primary explants were plated in medium 199 (GIBCO) supplemented with 4% FBS (HyClone), 10% chicken serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO). The cells that grew from

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Abbreviations: PPP, platelet-poor plasma; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; IGF, insulin-like growth factor.

*To whom reprint requests should be addressed.

the explants were passaged one time and then were subcultured on 96-well plates (Falcon 3004) at 3000 cells per well in DMEM supplemented with 10% FBS. After five days, the monolayers were washed and test factors were added with 0.2 ml of DMEM containing 0.5 μCi of [^{3}H]thymidine and 1% PPP; then [^{3}H]thymidine incorporation was determined after a 36-hr incubation as described previously. Mouse embryo fibroblasts were obtained from 18-day fetal BALB/c mouse (skin) and were grown in DMEM/10% FBS/10 mM glutamine (Sigma)/penicillin (100 units/ml)/streptomycin (100 μg/ml). Cultures between the third and fifth passages were used for all experiments.

Human fibroblasts were purchased from the Human Mutant Genetic Cell Respository (Camden, NJ). They were maintained in stock cultures in Eagle's minimum essential medium (MEM) (GIBCO) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% bovine serum (Colorado Serum, Denver, CO). They were passaged every 7 days using a passage dilution ratio of 1:4. Cultures between the fourth and fifth passages were plated at a density of 8000 cells per well on microtest plates for use in individual experiments. After 5 days, the cultures were washed twice with MEM then with 0.2 ml of MEM containing 1% PPP; 0.5 μCi of [^{3}H]thymidine and test substances were added, and [^{3}H]thymidine incorporation was determined after 36 hr as described previously.

To determine if the binding protein could potentiate the cell growth response to IGF-I, human fibroblasts or smooth muscle cells were plated at densities of 15,000 cells per cm^2 in 24-well plates (Falcon 3003) containing 1.0 ml of DMEM plus 10% FBS (smooth muscle) or MEM plus 10% calf serum (fibroblasts). After 2 hr, to allow for cell attachment, the media were aspirated and 1.0 ml of media containing 1% PPP was added. After a 12-hr incubation to allow the cells to become quiescent, the media were removed and replaced with 1.0 ml of MEM containing 1% PPP and the test substances. After 48 hr of incubation, the cultures were exposed to 1.0 ml of 0.5% trypsin/0.03% EDTA for 10 min at 37°C. This was removed and added to 9.0 ml of 0.15 mM NaCl, and the cell number was determined using a particle data counter (Coulter, Model ZBI).

Purification of Growth Factors. Human IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA). Porcine insulin (lot PJ5682) was a gift from Eli Lilly. The IGF binding protein was purified by a modification of a previously published method (11). The modified procedure substituted DEAE-cellulose chromatography as the second purification step. The peak that eluted with 100 mM NaCl was further purified by reverse-phase HPLC (C_4 column). The purification was monitored using a specific assay that measures IGF binding capacity (10). Ten microliters of each column fraction was incubated with 40,000 cpm of ^{125}I-labeled IGF-I (150 μCi/μg) (14) for 60 min at 22°C in 0.1 M Hepes (Sigma)/0.1% bovine serum albumin (Sigma)/0.1% Triton X-100/44 mM $NaCO_3$/0.02% NaN_3, pH 6.0 (250 μl volume). Bound and free ^{125}I-labeled IGF-I were separated by adding 1% immune serum globulin and 500 μl of 25% polyethylene glycol (M_r, 8000). The mixture was centrifuged at 8000 × g for 10 min, and the pellet was washed with 6.25% polyethylene glycol. The final product was pure as determined by polyacrylamide gel electrophoresis using silver staining and by amino acid sequence analysis (15).

RESULTS

The binding protein that had been purified from human amniotic fluid was incubated with quiescent porcine aortic smooth muscle cell cultures. This cell type was chosen because it does not have IGF binding protein that is adherent to its cell surface (10). Addition of the IGF-I binding protein alone resulted in minimal stimulation of [^{3}H]thymidine incorporation (Fig. 1*A*). Addition of IGF-I (20 ng/ml) or insulin (10 μg/ml) resulted in only 15 and 38% increases in [^{3}H]thymidine incorporation, respectively (Fig. 1*A*). In contrast, addition of IGF-I (20 ng/ml) plus the pure binding protein (100 ng/ml) resulted in a 4.4-fold stimulation (compared to binding protein alone), a potentiation that exceeded the cellular response to 10% human serum. At concentrations of 10 μg/ml, insulin binds to the type I IGF receptor but not to the binding protein (10). When these concentrations of insulin were added with the binding protein, there was no potentiation of the cellular replication response, indicating that activation of the binding protein effect was specific for IGF-I.

To determine if cell types from different species might respond to the IGF-I binding protein in a similar manner, the effect of this protein plus IGF-I was tested using chicken and mouse embryo fibroblast cultures. These cell types were chosen because the chicken cells do not synthesize an IGF binding protein, but they do possess type I IGF receptors. The mouse cells secrete a different form of IGF binding protein (≈22 kDa), and neither cell type has IGF binding protein on their cell surfaces. Addition of pure IGF binding protein resulted in only minimal changes in [^{3}H]thymidine incorporation in either cell type (Fig. 1 *B* and *C*). In contrast, the binding protein at 100 ng/ml plus IGF-I showed marked potentiation of the cellular response to IGF-I. The chicken fibroblasts were particularly sensitive; 81% of the maximal response induced by 10% human serum was reached with only 5 ng of IGF-I per ml (Fig. 1*B*). The mouse fibroblasts were also sensitive to the effects of the coincubation of IGF binding protein and IGF-I (Fig. 1*C*). Neither cell type responded to insulin plus IGF binding protein, indicating that the binding protein–IGF-I complex had to be formed to achieve the maximal stimulation of [^{3}H]thymidine incorporation.

To determine if human fibroblasts, a cell type that synthesizes the IGF binding protein, could also respond to exogenously added peptide, quiescent human fibroblast cultures were prepared as described in Fig. 2. Addition of increasing concentrations of IGF-I resulted in an increase in [^{3}H]thymidine uptake into DNA that was maximal at 20 ng/ml and was 82% greater than control cultures that were exposed to 1% PPP alone. The enhanced response to IGF-I alone compared to chicken or mouse fibroblasts may have been due to endogenously secreted IGF-I binding protein. Addition of the IGF binding protein alone resulted in no increase in DNA synthesis. However, when this protein (100 ng/ml) was added with increasing concentrations of IGF-I, DNA synthesis was augmented to a level that was equal to that obtained when 10% human serum was added. In separate studies, we have determined that these cells synthesize and secrete the IGF binding protein into media (16) and that this protein can attach to the cell surface (10). However, fibroblasts only secrete sufficient binding protein to reach media concentrations of 2–5 ng/ml, and since 100 ng/ml was added to these cultures, this difference probably accounts for the additional increase in [^{3}H]thymidine incorporation that was present.

To determine if these responses were dependent upon the concentration of the binding protein, increasing concentrations of binding protein (1–100 ng/ml) plus a constant concentration of IGF-I (10 ng/ml) were incubated with smooth muscle cell cultures. The cultures responded to binding protein at 2 ng/ml with a significant increase in [^{3}H]thymidine incorporation, but a maximal effect was not obtained until 100 ng/ml was used (Fig. 3). Since cultured fibroblasts secrete concentrations of IGF binding protein in the range of 2–5 ng/ml, this result indicates that the fibroblasts probably were not secreting a quantity of binding

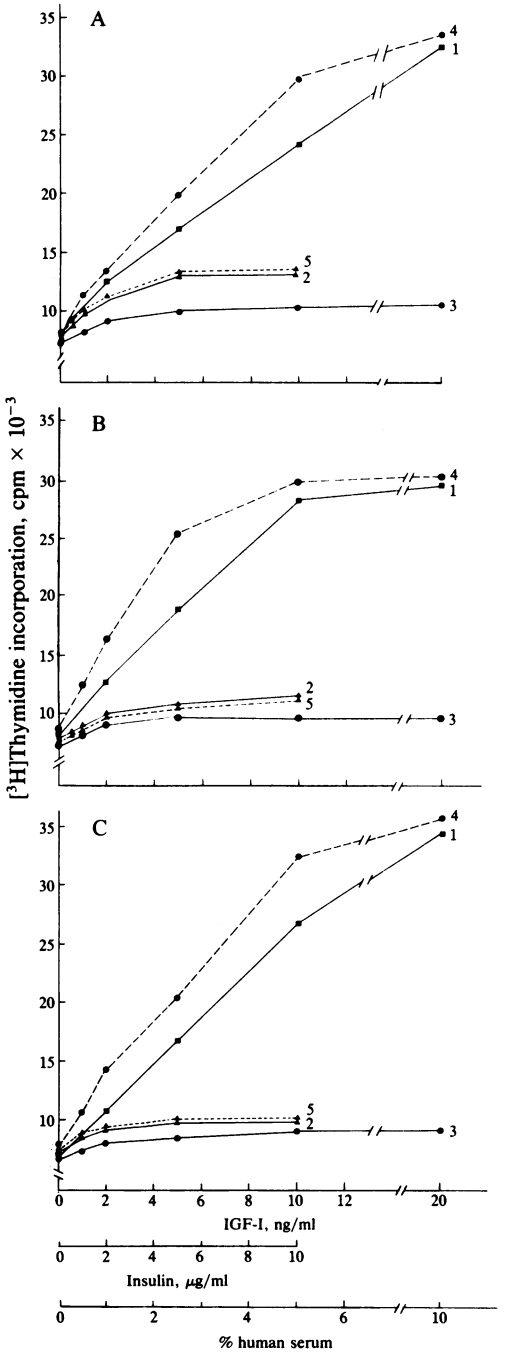

FIG. 1. Potentiation of DNA synthesis by porcine aortic smooth muscle cells and also chicken and mouse embryo fibroblasts by IGF-I and the IGF binding protein. (*A*) Smooth muscle cells were subcultured on 96-well microtest plates at a plating density of 8000 cells per well, and 5 days were allowed for the cells to become quiescent. Prior to the analysis, the cultures were washed twice with serum-free DMEM; then test substances were added in 0.2 ml of DMEM supplemented with 0.5 μCi of [³H]thymidine and 1% PPP. (*B*) Chicken embryo fibroblasts (second passage) were subcultured on 96-well microtest plates (Falcon 3004) at a plating density of 3000 cells per well in DMEM supplemented with 10% FBS. Five days after plating, the quiescent monolayers were exposed to the test factors listed plus 0.5 μCi of [³H]thymidine per ml and 1% PPP. (*C*) Mouse embryo fibroblast cultures between the third and fifth passages were used, and the experiments were performed as described for the chicken embryo fibroblasts except that the cells were plated at 8000 cells per well. Cultures were exposed to increasing concentrations of human serum (■—■, curves 1), insulin (▲—▲, curves 2), IGF-I (●—●, curves 3), IGF-I plus binding protein at 100 ng/ml (●—●, curves 4), or insulin and binding protein at 100 ng/ml (▲---▲, curves 5) for 36 hr, and [³H]thymidine incorporation was quantitated (13). Results are expressed as means of triplicate cultures.

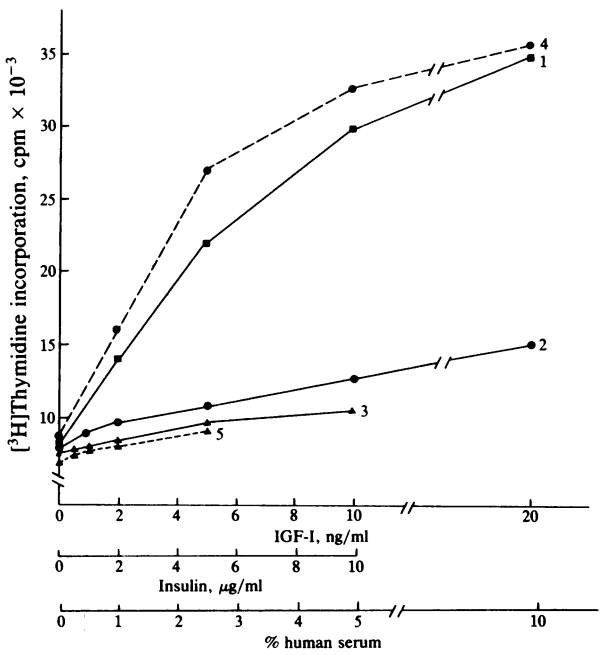

FIG. 2. Stimulation of DNA synthesis by the IGF binding protein in cultured human fibroblasts. Cells between the fourth and eighth passages were subcultured in 96-well microtest plates in MEM supplemented with 10% calf serum at a density of 8000 cells per well. Five days after plating, the cultures were washed with serum-free MEM; then 0.2 ml of MEM containing 0.5 μCi of [³H]thymidine, 1% PPP, and the stated concentrations of test substances were added. Increasing concentrations of human serum (■—■, curve 1), IGF-I (●—●, curve 2), insulin (▲—▲, curve 3), IGF-I plus binding protein at 100 ng/ml (●—●, curve 4), or insulin plus binding protein at 100 ng/ml (▲---▲, curve 5) were added to quiescent human fibroblast monolayers, and after a 36-hr incubation, DNA synthesis was determined. Results are expressed as means of triplicate cultures.

protein sufficient to achieve a maximal DNA synthesis response.

To determine if cells that were stimulated by IGF-I plus the binding protein could traverse the full cell cycle, smooth muscle cells and human fibroblasts were plated at a low density and made quiescent by serum deprivation. After 12 hr, the binding protein and IGF-I or insulin were added to test cultures, and the fibroblasts were incubated for 48 hr before the cell number was determined. Addition of IGF-I or insulin alone resulted in 3 and 11% increases in smooth muscle cell number, whereas the binding protein plus IGF-I (10 ng/ml) effected a 2.4-fold increase. This increase was greater than that induced by 10% human serum (Fig. 4*A*). Similar results were obtained with human fibroblasts, but the effect of IGF-I alone was greater (Fig. 4*B*). IGF-I alone resulted in a 31% increase, whereas the combination of binding protein plus IGF-I gave a 2.1-fold increase in cell number after 48 hr.

DISCUSSION

These results indicate that the addition of the IGF-I binding protein to cells exposed to IGF-I markedly facilitates the cell growth response of smooth muscle cells and fibroblasts to this growth factor. This response is specific for IGF-I since the combination of insulin with this protein did not result in enhancement of the growth response to this factor. Since the response to IGF-I plus binding protein is much greater than to IGF-I or binding protein alone, this suggests that the complex of both proteins is required for maximal growth stimulation. This requirement could provide a mechanism for tissue specific stimulation of growth by IGF-I since the presence of the binding protein in the microenvironment

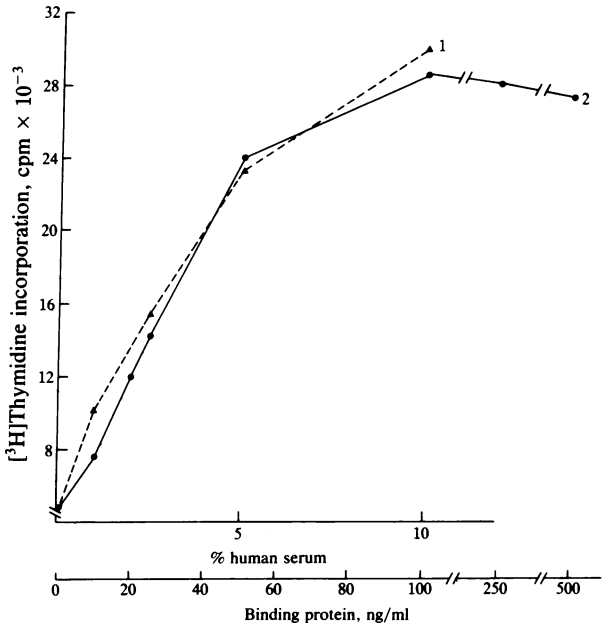

FIG. 3. Concentration-dependent increases in smooth muscle cell DNA synthesis in response to the binding protein. Cultures were exposed to increasing concentrations of human serum (▲---▲, curve 1) or the pure IGF-I binding protein (10–500 ng/ml), IGF-I (10 ng/ml) and 1% human PPP (●—●, curve 2). Smooth muscle cell cultures were prepared, and [3H]thymidine incorporation was determined as described in Fig. 1. Results are expressed as the mean [3H]thymidine incorporation of triplicate cultures.

could markedly augment the cellular response, and cell types that secrete the binding protein might be able to respond to IGF-I preferentially. This finding indicates that IGF binding proteins may not only serve a transport function, as has been postulated, but in cases where they can adhere to cell surfaces the IGF binding proteins may actually facilitate the cell growth responses to IGF-I directly. This might be achieved by altering IGF-I degradation or by increasing its binding to the type I receptor.

Our laboratory has previously shown that human fibroblasts synthesize and secrete an IGF binding protein that can be specifically immunoprecipitated by an antibody to the human amniotic fluid protein. Following its synthesis, the fibroblast protein interacts with the cell surface, and its presence on the cell surface is associated with an increase in cell-associated IGF-I (10). Addition of increasing concentrations of IGF-I results in increased cell association of IGF-I due to enhanced binding to both cell surface binding protein and the type I IGF receptor. Inhibition of synthesis of the binding protein by coincubation with cycloheximide completely eliminates this enhanced association of IGF-I with the cell surface. The above data support the concept that there is an interaction between the cell surface concentration of the binding protein and enhancement of IGF-I binding to both its receptor and the binding protein. This enhancement of IGF-I binding could be directly related to the marked augmentation in DNA synthesis that is induced by the IGF-I–binding protein complex as noted in these studies. It is possible that this increase in the amount of IGF-I that is associated with the cell surface and the concomitant increase in binding to the type I receptor activate a major cellular growth response to IGF-I and that under these conditions mesenchymal cell growth is partially independent of the usual requirements for other mitogens, such as platelet-derived growth factor or epidermal growth factor.

These findings suggest that cell types that secrete this binding protein might be able to augment their response to IGF-I in an autocrine or paracrine manner. Control of synthesis and secretion of the protein could function as a mechanism to control cell proliferation in the microenvironment and to locally modulate the response to IGF-I even at a time when blood and extracellular fluid concentrations of this growth factor remained constant. Although plasma concentrations of the binding protein have been shown to be age and estrogen dependent (17), control of its secretion by tissues has not been studied in detail. We have demonstrated

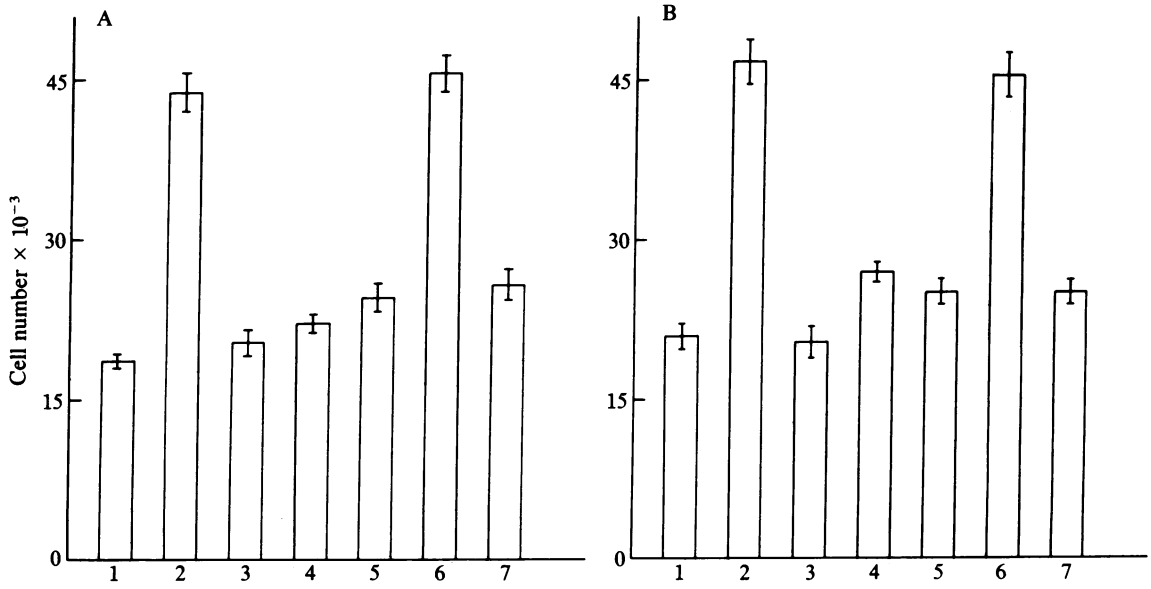

FIG. 4. Growth of cultured fibroblasts and smooth muscle cells in response to IGF-I and the IGF binding protein. Porcine smooth muscle cells (*A*) and human fibroblasts (*B*) were plated at 15,000 cells per well on 24-well plates (Falcon 3004) in MEM plus 10% calf serum (fibroblasts) or DMEM plus 10% fetal calf serum (smooth muscle cells). After 2 hr, the media were changed to MEM or DMEM containing 1% PPP. Following an additional 12-hr incubation, the media were removed and test reagents were added to 0.5 ml of DMEM or MEM containing 1% PPP. Following a 48-hr incubation, cell number was determined. The results are expressed as the mean ± 1 SD of quadruplicate cultures. Bars: 1, 1% PPP; 2, 10% human serum; 3, binding protein (100 ng/ml); 4, IGF-I (10 ng/ml); 5, insulin (10 μg/ml); 6, binding protein (100 ng/ml) plus IGF-I (10 ng/ml); 7, binding protein (100 ng/ml) plus insulin (10 μg/ml).

previously that exposure of BALB/c 3T3 fibroblasts to platelet-derived growth factor can induce an increase in IGF-I binding to the cell surface (14). Since this change could have been due to an increase in the amount of IGF binding protein that was present, we have measured the cell surface concentration of the binding protein after exposure to platelet-derived growth factor. We found, however, that its level on the fibroblast surface was not increased by exposure to platelet-derived growth factor or epidermal growth factor (D.R.C., M. Dehoff, and S. L. S. Drop, unpublished results). Recently Rutanen *et al.* demonstrated that progesterone increases the IGF binding protein synthesis in human decidual tissue explants (18). It is possible, therefore, that local synthesis and release of the IGF binding protein may be under hormonal regulation in specific tissues.

The exact mechanism by which the binding protein–IGF-I complex activates DNA synthesis was not addressed by these studies. It is possible that the complex associates directly with the membrane to activate transmembrane signal mechanisms or that it can activate these processes only in cooperation with the type I receptor. Alternatively, the IGF-I–binding protein complex might be directly internalized and act at an intracellular site. Regardless of the mechanism of transmembrane signaling, it is possible that this complex could induce the synthesis of a specific growth regulatory protein. Recently the type β transforming growth factor has been shown to induce c-sis and c-myc expression in AKR-2B cells in culture (19). Since many growth regulatory proteins are believed to control early events in cell cycle progression, it will be of interest to determine if this complex can enhance their expression.

Other growth factors have been shown to associate with binding proteins (20, 21) either during secretion or transport in plasma; however, none of the other growth factor binding proteins have been shown to augment the cellular growth response to these mitogens. Since many of the binding proteins may not have been tested, however, this possibility has not been rigorously excluded. During the purification of the IGF binding protein, we noted that impure material inhibited the growth response to IGF-I, and it was only when material was obtained in pure form that its stimulatory activity was noted. Our observation that a pure form of this protein is required to detect this biologic response may help to explain data of others showing that similar IGF binding proteins purified from plasma or cellular conditioned media inhibit IGF action. Zapf *et al.* (3) found that an impure preparation of the IGF binding protein purified from plasma inhibited insulin-like actions of IGF-I. Likewise Drop *et al.* (8), by using impure human amniotic fluid protein, showed inhibition of cartilage sulfation by IGF-I. A possible explanation for this difference between pure and impure preparations of the IGF-binding protein has been provided by our recent observation that human amniotic fluid contains two forms of the binding protein and that one form inhibits IGF-I-stimulated DNA synthesis (15). Knauer and Smith have used a homogeneous preparation of rat liver cell-derived IGF binding protein, however, and have found it to inhibit DNA synthesis in chicken embryo fibroblasts (12). This species difference may be important since the rat protein does not crossreact with antibody to the human protein and has a distinct amino terminal sequence (11, 22). Our laboratory has noted that the rat form of the binding protein does not associate with the chicken embryo or human fibroblast cell surfaces, and this observation supports the hypothesis that cell surface association may be required for the biologic response noted herein.

The presence of an alternative mechanism for activating cell growth suggests possible approaches to the analysis of pathophysiologic conditions. Many types of dwarfism are associated with normal blood IGF-I concentrations, but they might be due to a deficiency of the IGF binding protein (23). Likewise, some syndromes such as Beckwith–Weideman (24) that are associated with macrosomia but normal IGF-I concentrations could be due to a generalized increased synthesis of this protein. Of greater interest is the possibility that local synthesis of this protein by fibroblasts or other cell types could augment the local response to IGF-I. This could occur in response to injuries, such as wounding or atherosclerosis, where a localized proliferative response occurs but is confined to a small area. By this mechanism, the cells that are secreting increased concentrations of the binding protein could divide in the presence of normal ambient IGF-I levels while surrounding tissues that are exposed to equal IGF-I concentrations might be less sensitive to its effects.

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