# ANIMAL EXPERIMENTATION

## Immunohistochemical Analysis of Insulin-like Growth Factor–Binding Proteins -1, -2, and -3 in Implantation Sites of the Mouse[1]

MARK A. DAMARIO,[2,5] HUNG-CHING LIU,[2] CAROL ANN MELE,[2] MARCELO G. HORENSTEIN,[3,4] and ZEV ROSENWAKS[2]

*Submitted: January 5, 1998*
*Accepted: April 1, 1998*

***Purpose:*** *Our purpose was to analyze potential interactions between the embryo and the maternal endometrial interface in vivo by analyzing immunolocalization of insulin-like growth factor–binding proteins (IGFBPs) -1, -2, and -3 in implantation sites of the mouse.*
***Methods:*** *Six-week-old $B_6D_2F_1$ female mice underwent superovulation followed by mating and sacrifice at timed intervals. Formalin-fixed paraffin-embedded tissue was used for avidin–biotin immunocytochemical localization of IGFBPs utilizing standard methodology.*
***Results:*** *Immunostaining at 1.5 days post coitum revealed light staining in the epithelial glandular cells and faint staining in decidual stroma for both IGFBP-1 and IGFBP-2. At 7.5–10.5 days post coitum, there was moderate–dense immunostaining in the decidualized stromal cells at the implantation site for all three IGFBPs, whereas light immunostaining was seen in nonimplantation site decidua.*
***Conclusions:*** *Compartmentalization of immunostaining for IGFBP-1, -2, and -3 within decidualized stroma suggests that these proteins may be regulated by trophoblastic and/or embryonic signals.*

[1] Presented at the 43rd Annual Meeting of the Society of Gynecologic Investigation, Philadelphia, Pennsylvania, March 20–23, 1996.

[2] Center for Reproductive Medicine and Infertility, Department of Obstetrics and Gynecology, Cornell University Medical College, New York, New York.
[3] Department of Pathology, Cornell University Medical College, New York, New York.
[4] Current address: Department of Pathology, Duke University Medical Center, Durham, North Carolina.
[5] To whom correspondence should be addressed at Mayo Clinic, 200 First Street SW, Rochester, Minnesota 55905.

**KEY WORDS:** decidua; immunohistochemistry; implantation; insulin-like growth factor–binding proteins.

## INTRODUCTION

There is increasing evidence that there is maternal–embryonic communication that occurs during the implantation process. One source of communication may be through the insulin-like growth factor (IGF) system, which is felt to play important roles in placentation and the decidual response. This system is composed of two ligands (IGF-I and IGF-II), specific receptors (type I and type II receptors), and a family of proteins called the IGF-binding proteins (IGFBPs), which regulate the availability of IGFs to target cells (1). IGFBPs have been found to have various modulating actions on IGFs both in vitro and in vivo. For example, IGFBPs have been shown to prolong the biological half-life of the IGFs by preventing degradation, to inhibit the action of IGFs by forming an inactive complex, to potentiate the actions of the growth factors by facilitating transport to cell surface receptors, and to sequester IGFs in extracellular matrix (2,3). To date, six IGFBPs (IGFBP-1, -2, -3, -4, -5, and -6) have been isolated. We, as well as others, have shown that production of IGFBPs by human endometrial stromal cells cultured in vitro is hormonally regulated (4–8). In addition, the secretion of IGFBPs by endometrial stromal cells appears to be stimulated by the presence of embryos (9).

A cell-specific and temporal pattern of IGF-I mRNA expression has been shown in the periimplantation


1058-0468/98/0900-0513$15.00/0 

mouse uterus (10). Preimplantation mouse embryos also express IGFs and their receptors. IGF-II and the type II IGF receptor are expressed in two-cell mouse embryos, and the type I IGF receptor can be detected at the eight-cell stage (11). IGF-I mRNA has not been demonstrated in the preimplantation mouse embryo. Maternally derived IGF-I, however, is available to mouse embryos within reproductive tract fluids (12). Optimal mouse preimplantation embryonic development appears to require both autocrine and paracrine growth factor interactions. IGF-I has been noted to stimulate growth of mouse preimplantation embryos in vitro (13). Targeted disruption of one of the IGF-II alleles results in germ-line transmission of an inactivated IGF-II gene and resultant progeny that are approximately half the size of their wild-type littermates (14). In vitro experiments using mouse placental extracts suggest that IGF-I stimulates proliferation and migration of ectoplacental cone cells, whereas IGF-II transforms these cells into trophoblastic giant cells (15). In vitro experiments using human cells have shown that there is a gradient of IGF-II mRNA abundance in trophoblast columns, with the greatest levels expressed at the invading front (16). The evidence from different animal systems, therefore, suggests that IGFs play a role in embryonic development and trophoblast functioning. The production of maternal IGFBPs, primarily from the decidua, appears to play a key role in the regulation of these processes.

IGFBP-2, -3, -4, -5, and -6 mRNA has been detected in uterine horns of mice during the preimplantation phase of gestation (17). By day 6, shortly after implantation, IGFBP-4 mRNA signals have been noted to produce intense bands at implantation sites and adjacent decidua (18). IGFBP-4 signals were absent in uterine tissue between the implantation sites in this latter study. In the present experiment, we proposed to examine further the roles of IGFBPs in the implantation process by studying the temporal and spatial distribution of IGFBP -1, -2, and -3 in implantation sites of the mouse.

## MATERIALS AND METHODS

### Animals

For immunohistochemical studies, tissues were obtained from 6-week-old $B_6D_2F_1$ mice; uteri were extracted from a minimum of four mice for each day of gestation studied. Females underwent superovulation with pregnant mare serum gonadotropin (PMSG; Sigma, St. Louis, MO), 5 IU intraperitoneally, followed by human chorionic gonadotropin (hCG; Sigma), 5 IU intraperitoneally, 48 hr later. The mice were mated with normal fertile males of the same species from 1700 to 0800. After mating, animals exhibiting copulation plugs were sacrificed at fixed intervals. On appropriate gestational days, mice were killed by cervical dislocation. Uteri were rapidly removed and fixed in freshly prepared 4% paraformaldehyde, followed by tissue embedding and processing. Early implantation sites were then identified from stained sections. Five-micrometer adjacent sections immediately proximate to the identified implantation sites were then cut using a rotary microtome from paraffin-embedded tissue.

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and a protocol approved by the Cornell University Medical College Animal Care and Use Committee.

### Immunological Reagents

Three primary antisera were used, all made in the rabbit. A polyclonal anti-human IGFBP-1, (UBI, Lake Placid, NY), which was purified from sera of animals immunized with IGFBP-1 purified from human amniotic fluid, was used. A polyclonal antibovine IGFBP-2 (UBI), which was purified by immunoaffinity and high-performance liquid chromatography (HPLC) from Madin Darby bovine kidney cells, was used. A polyclonal anti-human IGFBP-3 (UBI), which was purified from sera of animals immunized with purified

**Table I.** Immunohistochemical Results[a]

| | Day 1.5 | | Days 7.5–8.5 | | Days 9.5–10.5 | |
|---|---|---|---|---|---|---|
| Antibody | Glandular epithelium | Decidualized stroma | I.S. decidua | Non-I.S. decidua | I.S. decidua | Non-I.S. decidua |
| IGFBP-1 | + | Faint | ++ | Faint to + | +++ | 0 to ++ |
| IGFBP-2 | + | Faint | ++ to +++ | + | +++ | 0 to ++ |
| IGFBP-3 | 0 | 0 | ++ | Faint to + | +++ | 0 to ++ |
| Rabbit IgG | 0 | 0 | 0 | 0 | 0 | 0 |

[a] I.S., implantation site.

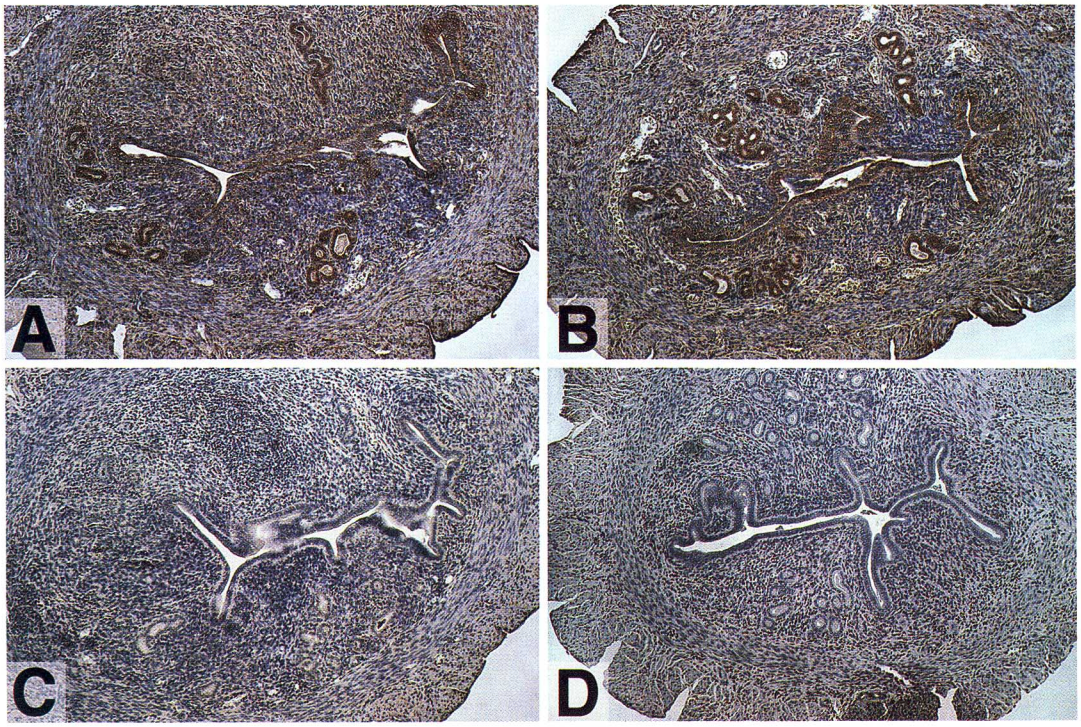



**Fig. 1.** Immunohistochemical staining of mouse uterine horns at the preimplantation stage (1.5 days postcoitum). (A) IGFBP-1; (B) IGFBP-2; (C) IGFBP-3; (D) control (rabbit IgG). Original magnification, ×100.

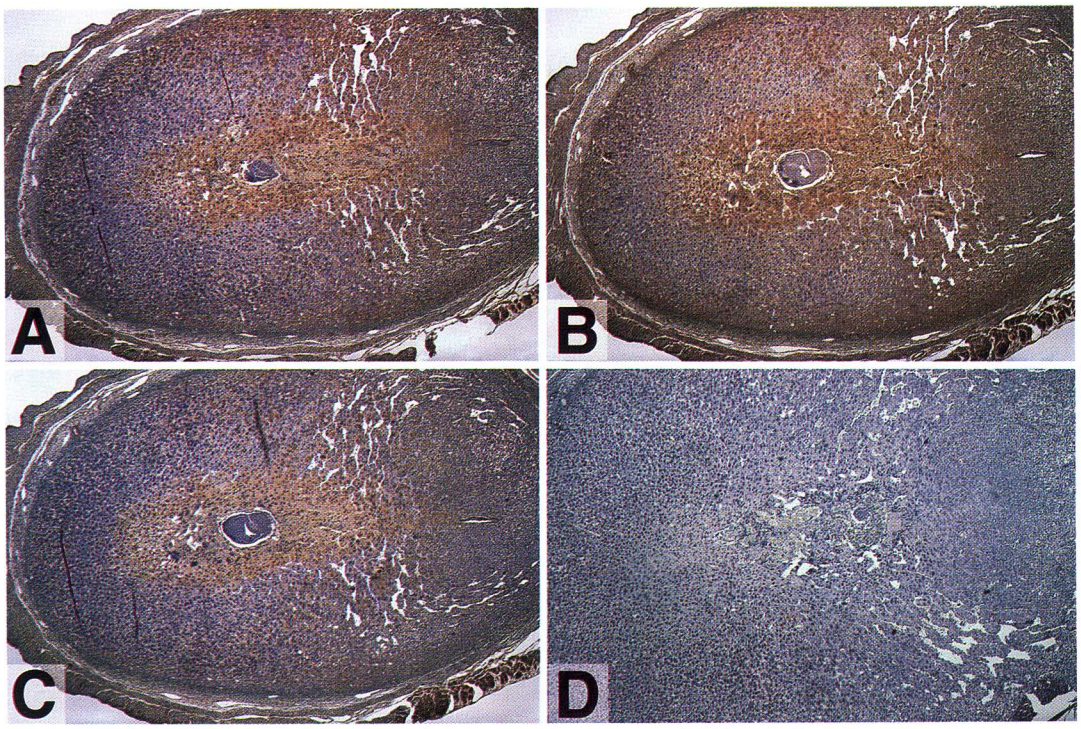



**Fig. 2.** Immunohistochemical staining of early mouse implantation sites (7.5–8.5 days postcoitum). (A) IGFBP-1; (B) IGFBP-2; (C) IGFBP-3; (D) control (rabbit IgG). Original magnification, ×40.

human recombinant IGFBP-3 was used. A secondary biotinylated antibody (Vector Laboratories, Burlingame, CA), made in goat, which recognizes both heavy and light IgG chains of rabbit, was used. The three primary antibodies have been successfully used in our laboratory to demonstrate immunocytochemical staining of IGFBPs in murine embryos (5).

### Immunohistochemistry Procedure

After dewaxing, sections were rehydrated and treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. Blocking of nonspecific antigenic sites was then undertaken with 1.5 to 2.5% normal goat sera. Primary antibody was then layered onto the slides for 1 hr at room temperature. Initial titration experiments revealed that optimal titers of primary antibodies were 1:200 for paraformaldehyde-fixed tissue. After thorough washing with 0.1% Tween–phosphate-buffered saline (T-PBS), the secondary biotinylated antibody was incubated on the slides for 30 min. Avidin–biotin immunocytochemical localization of IGFBPs was then carried out using the ABC Elite Vectastain kit (Vector Laboratories). Diaminobenzidine tetrahydrochloride (DAB) was the chromogen substrate. Sections were lightly counterstained with Gill's hematoxylin (Sigma). Control sections included (a) deletion of the primary antibody with substitution of T-PBS buffer, and (b) purified rabbit immunoglobulin (Ig)G at a concentration of 1 μg/ml. In addition, we performed two experiments in which slides from animals 9.5–10.5 days postcoitum were coincubated with primary antibody and 200 ng of commercially available recombinant human IGFBP-1 and -3 (UBI), respectively.

Immunostaining was graded from the lowest (0) to the highest (+++) intensity for each section studied and was evaluated independently by two investigators blinded to the type of primary antibody used.

## RESULTS

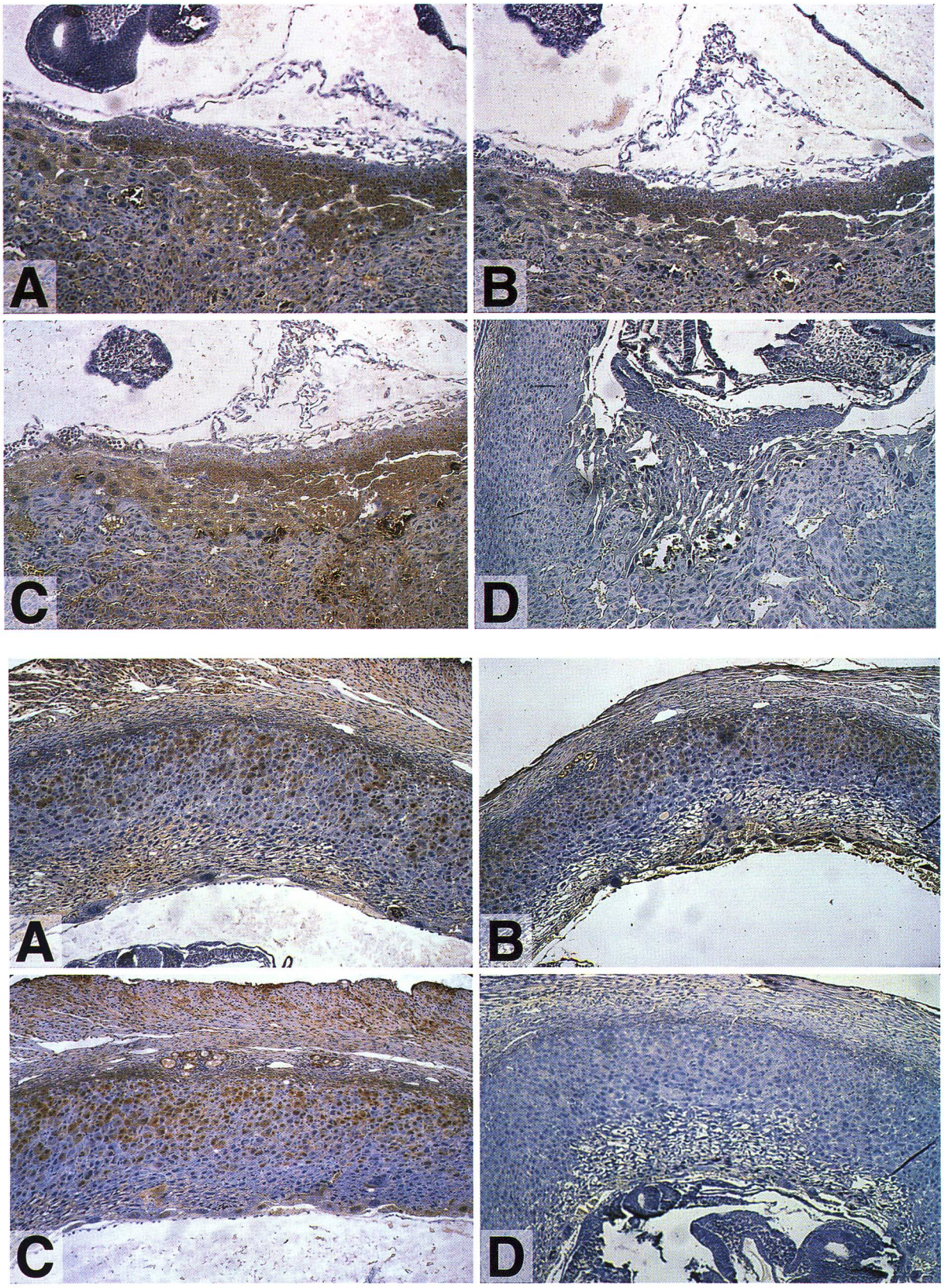
Immunostaining for the three specific IGFBPs exhibited many similarities (Table I). At 1.5 days postcoitum (preimplantation stage), there was light staining in the epithelial glandular cells and faint staining in the decidual stroma for IGFBP-1 and -2 (Fig. 1). No identifiable staining could be ascertained for IGFBP-3 at this stage. For both IGFBP-1 and IGFBP-2 there appeared to be slightly more intense staining in the glandular epithelium rather than the luminal epithelium at this stage. Attempts to localize the very earliest implantation sites (ectoplacental cone) at 4.5–6.5 days postcoitum were not consistently successful. Therefore, statements about the immunostaining patterns at this specific stage were not made. At 7.5–8.5 days postcoitum (advanced egg cylinder–primitive streak stage), moderately dense immunostaining began to appear in the decidualized stromal cells at the implantation site for all three IGFBPs, whereas faint–light immunostaining was seen in nonimplantation site decidua (Fig. 2). For each IGFBP tested, there appeared to be a halo of intense decidual stromal staining localized immediately adjacent to the conceptus and implantation site. At 9.5–10.5 days postcoitum (early somite stage), a marked band of dense immunostaining comprised of the maternal surface of the placenta and the adjacent decidua could be noted for all three IGFBPs (Fig. 3). The nonimplantation site decidua was characterized by only light staining of cells on the luminal surface, with moderate–dense staining in some deeper cells (Fig. 4). Control sections without a primary antibody or with rabbit IgG from a nonimmunized animal did not result in any identifiable immunostaining at any stage tested. The two experiments involving primary antibodies coincubated with their respective commercially available antigens resulted in significantly diminished, although not completely eliminated, immunostaining.

## DISCUSSION

There were many similarities in the immunostaining results for the three IGFBPs. During the preimplantation phase, however, no immunostaining of the uterine horns was noted for IGFBP-3. This is similar to previous findings with IGFBP-4, with an absence of in situ hydridization signals in the uterine horns of ani-

**Fig. 3.** (*opposite, top*) Immunohistochemical staining of later mouse implantation sites (9.5–10.5 days postcoitum). (A) IGFBP-1; (B) IGFBP-2; (C) IGFBP-3; (D) control (rabbit IgG). Original magnification, ×100.

**Fig. 4.** (*opposite, bottom*) Immunohistochemical staining of decidualized stroma in locations not directly at the implantation sites (9.5–10.5 days postcoitum). (A) IGFBP-1; (B) IGFBP-2; (C) IGFBP-3; (D) control (rabbit IgG). Original magnification, ×100.


A
B
C
D
A
B
C
D

mals noted during the preimplantation phase (18). IGFBP-1 and IGFBP-2 immunostaining, however, could be seen during the preimplantation phase in our experiments. Staining was most prominent in the uterine epithelium at this stage for both binding proteins, although the glandular epithelium in each case appeared more stained than the luminal epithelium. There was only faint staining of the stroma at this stage for both IGFBPs. With subsequent implantation and the presence of an embryo, immunostaining for all three IGFBPs increased. Of significance was that the immunostaining was compartmentalized such that the most marked staining was adjacent to the embryo/implantation site, whereas lesser staining was noted in areas more distant to the implantation site.

Because of the similarity in staining patterns of the three IGFBPs we performed limited experiments to evaluate antibody specificity further. One slide was prepared for each of the primary antibodies and their commercially available respective antigens used at a concentration that was felt to represent significant antigen excess. The absence of commercially available purified rhIGFBP-2 did not allow us to test this respective antibody. The two experiments performed, however, resulted in significantly less immunostaining for antibodies to IGFBP-1 and -3, respectively. These results, in addition to personal communication with the antibody manufacturer that cross-reactivity of these antibodies to any other IGFBP is less than 2% for usual working dilutions, assured us that our results were most likely specific for each IGFBP antibody tested. Further optimization of our experimental conditions (i.e., antigen:antibody titers, incubation conditions, etc.) may be necessary to attain complete neutralization of all immunostaining.

While our methodology does not permit distinction of the precise source of the protein located at the maternal–embryonic interface, these three IGFBPs appear to play a role in the implantation process of the mouse. Work in other species has revealed some similarities and some differences. Sadek *et al.* studied the immunocytochemical localization of IGFBP-1 in the rat during early pregnancy (19). Immunoreactive IGFBP-1 was first detected from days 5–6 of pregnancy onward in the luminal and glandular epithelium, with immunoreactivity most intense in the glandular epithelium. However, IGFBP-1 was not detected in rat decidua. The latter results were somewhat in contrast with those of Croze *et al.*, who found significantly increased expression of IGFBP-1 in the rat uterus during decidualization (20). Boomsma *et al.* examined the immunocytochemical localization of IGFBP-1 in implantation sites of the cat (21). Specific immunostaining was observed in surface and glandular epithelium of the nonimplantation site endometrium throughout pregnancy. The placenta and the junctional zone immediately beneath the placenta were very strongly stained. In the endometrium further below the junctional zone, there was less intense specific staining. Tarantino *et al.* evaluated the regulation of IGFBPs in the baboon uterus during early pregnancy (22). Immunoprecipitation followed by ligand blot analysis confirmed that IGFBP-1 and -2 were the predominant products of the endometrium and decidua, whereas IGFBP-3 was synthesized by the placenta. Immunocytochemical staining was successful only for IGFBP-1. Intense glandular epithelial staining for IGFBP-1 was present on days 18, 25, and 32. Stromal staining for IGFBP-1 was initially present on day 25 and was present only in stromal cells that had intimate contact with trophoblastic cells. By day 32, IGFBP-1 expression was not limited to the endometrial–trophoblastic junction, but also extended to deeper stromal cells.

Much debate continues regarding the possible roles of IGFBPs at the maternal–fetal interface. There is some information that suggests that IGFBP-1 may play a role in limiting trophoblast invasiveness. Irwin *et al.* cocultured cytotrophoblasts with decidualized stromal cells (which make large quantities of IGFBP-1) and noted inhibition of cytotrophoblast invasion into the decidual cell multilayer (23). The addition of insulin resulted in restoration of cytotrophoblast invasion (presumably by suppression of IGFBP-1 secretion), whereas the addition of IGFBP-1 back to this culture inhibited invasion. An antibody to IGFBP-1 subsequently blocked the inhibitory effect on cytotrophoblast invasion that is produced by decidualized endometrial stromal cells. The precise mechanisms whereby IGFBP-1 inhibits cytotrophoblast invasion are not known. Perhaps the main action is through inhibition of IGF action at the maternal–embryonic interface. IGFBP-1 may also serve as an extracellular matrix protein which directly signals events in the trophoblast. IGFBP-1 has an RGD (Arg–Gly–Asp) sequence that enables binding to the $\alpha_5\beta_1$ integrin (fibronectin receptor), which is expressed on cytotrophoblast cell surfaces (1). On the contrary, Irving and Lala have recently reported that IGFBP-1 promotes rather than inhibits trophoblast migration using an in vitro assay system in which passaged trophoblast cells are observed in petri dishes (24). Trophoblast migration in this system, however, could be inhibited in the presence of $\alpha_5$ and $\beta_1$ integrin blocking antibodies, suggesting that (a) trophoblast migration requires the expression of $\alpha_5$ and $\beta_1$ integrin subunits and (b)

IGFBP-1 may be more likely to play a role in mediating trophoblast migration–related effects through its interaction with the RGD binding site of $\alpha_5\beta_1$ integrin.

On the other hand, very little is known about the potential roles of the other IGFBPs in the implantation process. Both IGFBP-1 and IGFBP-3 have the ability to potentiate the mitotic activity of fibroblast cells in vitro (25,26). IGFBPs could also possibly serve as reservoirs and regulate the release of IGFs into their local environment (27,28). It has been postulated that the IGFBPs may initially provide a stimulatory effect on enhancing stromal cell and/or trophoblast proliferation. Once placentation is established and the decidual process is complete, IGFBP functions may be modified to inhibit further trophoblast invasion. This is supported by studies that reveal that the degree of phosphorylation of IGFBP-1 in various tissues increases as pregnancy progresses in the human (29). Binding affinity for IGF-1 increases as IGFBP-1 becomes more phosphorylated and may result in less IGF-1 bioavailability (30).

## CONCLUSIONS

The periimplantation uterus is a site of significant synthesis of a number of IGFBPs in several species. There is frequent up-regulation of one or more of the IGFBPs during the periimplantation period. Immunohistochemical studies in the mouse reveal similar immunostaining patterns for IGFBP-1, -2, and -3. The most prominent findings are marked bands of dense immunostaining for all three IGFBPs occurring at the maternal–trophoblast interface of early implanting embryos. The roles that these IGFBPs play in the implantation process is not yet fully defined, although possible functions include control of trophoblast invasion and regulation of the decidual process. The mechanisms whereby IGFBPs act in this process are either through direct IGFBP effects or through modulation of IGF activity. The compartmentalization of the IGFBPs within the decidualized stroma suggests that these proteins may be in response to embryonic signals. Further research is needed to clarify the precise role of the IGFBPs in the implantation process.

## REFERENCES


1. Rosenfield RG, Lamson G, Pham H, Oh YM, Conover C, DeLeon D, Donovan SM, Ocrant I, Giudice LC: Insulin-like growth factor binding proteins. Rec Progr Horm Res 1991;16:99–159
2. Kelley KM, Oh Y, Gargosky SE, Gucev A, Matsumoto T, Hwa V, Ng L, Simpson DM, Rosenfeld RG: Insulin-like growth factor-binding proteins (IGFBPs) and their regulatory dynamics. Int J Biochem Cell Biol 1996;28:619–637
3. Le Roith D: Insulin-like growth factor receptors and binding proteins. Bailliere's Clin Endocrinol Metab 1996;10:49–73
4. Bell SC, Jackson JA, Ashmore J, Zhu HH, Tseng L: Regulation of insulin-like growth factor-binding protein-1 synthesis and secretion by progestin and relaxin in long term cultures of human endometrial stromal cells. J Clin Endocrinol Metab 1991;72:1014–1024
5. Giudice LC, Milkowski DA, Fielder PJ, Irwin JC: Characterization of the steroid-dependence of insulin-like growth factor-binding protein-2 synthesis and mRNA expression in cultured human endometrial stromal cells. Hum Reprod 1991;6:632–640
6. Tseng L, Gao J-G, Chen R, Zhu HH, Mazella J, Powell DR: Effect of progestin, antiprogestin, and relaxin on the accumulation of prolactin and insulin-like growth factor-binding protein-1 messenger ribonucleic acid in human endometrial stromal cells. Biol Reprod 1992;47:441–450
7. Giudice LC, Dsupin BA, Irwin JC: Steroid and peptide regulation of insulin-like growth factor-binding proteins secreted by human endometrial stromal cells is dependent on stromal differentiation. J Clin Endocrinol Metab 1992;75:1235–1241
8. Liu H-C, He Z-Y, Mele C, Damario M, Davis O, Rosenwaks Z: Hormonal regulation of expression of messenger RNA encoding insulin-like growth factor binding proteins in human endometrial stromal cells cultured *in vitro*. Mol Hum Reprod 1997;3:21–26
9. Liu H-C, Mele C, Catz D, Noyes N, Rosenwaks Z: Production of insulin-like growth factor binding proteins (IGFBPs) by human endometrial stromal cells is stimulated by the presence of embryos. J Assist Reprod Genet 1995;12:78–87
10. Kapur S, Tamada H, Dey SK, Andrews GK: Expression of insulin-like growth factor-1 (IGF-1) and its receptor in periimplantation mouse uterus, and cell-specific regulation of IGF-1 gene expression by estradiol and progesterone. Biol Reprod 1992;46:208–219
11. Rappolee DA, Sturm KS, Behrendtsen O, Schultz GA, Pedersen RA, Werb Z: Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. Genes Dev 1992;6:939–952
12. Heyner S, Shah N, Smith RM, Watson AJ, Schultz GA: The role of growth factors in embryo production. Theriogenology 1993;39:151–161
13. Harvey MB, Kaye PL: Insulin-like growth factor-I stimulates growth of mouse preimplantation embryos in vitro. Mol Reprod Dev 1992;31:195–199
14. DeChiara TM, Efstratiadis A, Robertson EJ: A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 1990;345:78–80
15. Kanai-Azuma M, Kanai Y, Kurohmaru M, Sakai S, Hayashi Y: Insulin-like growth factor (IGF)-I stimulates proliferation and migration of mouse ectoplacental cone cells, while IGF-II transforms them into trophoblastic giant cells in vitro. Biol Reprod 1993;48:252–261
16. Han VKM, Bassett N, Walton J, Challis JRG: The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: Evidence for IGF-IGFBP interactions at the feto-maternal interface. J Clin Endocrinol Metab 1996;81:2680–2693

17. Hahnel A, Schultz GA: Insulin-like growth factor binding proteins are transcribed by preimplantation mouse embryos. Endocrinology 1994;134:1956–1959
18. Markoff E, Henemyre C, Fellows J, Pennington E, Zeitler PS, Cedars MI: Localization of insulin-like growth factor binding protein-4 expression in the mouse uterus during the periimplantation period. Biol Reprod 1995;53:1103–1109
19. Sadek S, Unterman TG, Bell SC: Epithelial localization of insulin-like growth factor binding protein 1 in the uterus of the rat during pregnancy, deciduoma-bearing pseudopregnancy and hormone treatment. J Reprod Fertil 1994;101:67–75
20. Croze L, Kennedy TG, Schroedter IC, Friesen HG, Murphy LJ: Expression of insulin-like growth factor-I and insulin-like growth factor binding protein-1 in the rat uterus during decidualization. Endocrinology 1996;71:1995–2000
21. Boomsma RA, Mavrogianis PA, Fazleabas AT, Jaffe RC, Verhage HG: Detection of insulin-like growth factor binding protein-1 in cat implantation sites. Biol Reprod 1994;51:392–399
22. Tarantino S, Verhage HG, Fazleabas AT: Regulation of insulin-like growth factor-binding proteins in the baboon (*Papio anubis*) uterus during early pregnancy. Endocrinology 1992;130:2354–2362
23. Irwin JC, Dsupin BA, Giudice LC: Modulation of cytotrophoblast interaction with fibronectin and endometrial stromal cell cultures by insulin-like growth factor binding protein-1 (IGFBP-1). Presented at the Society for the Study of Reproduction, Sacramento, CA, July 1995
24. Irving JA, Lala PK: Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-β, IGF-II, and IGFBP-1. Exp Cell Res 1995;217:419–429
25. Lee PDK, Conover CA, Powell DR: Regulation and function of insulin-like growth factor binding protein-1. Proc Soc Exp Biol Med 1993;204:4–29
26. Sara VR, Hall K: Insulin-like growth factors and their binding proteins. Physiol Rev 1990;70:591–614
27. Blum WF, Jenne EW, Reppin T, Kietzmann K, Ranke MB, Bierich JR: Insulin-like growth factor I (IGF-I)-binding protein complex is a better mitogen than free IGF-I. Endocrinology 1989;125:766–772
28. Conover CA: Potentiation of insulin-like growth factor (IGF) action by IGF-binding protein-3: Studies of underlying mechanism. Endocrinology 1992;130:3191–3199
29. Koistinen R, Angervo M, Leinonen P, Seppala M: Phosphorylation of insulin-like growth factor-binding protein-1 from different sources. Growth Reg 1993;3:34–37
30. Jones JI, D-Ercole AJ, Camacho-Hubner C, Clemmons DR: Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: Effects on affinity for IGF-1. Proc Natl Acad Sci USA 1991;88:7481–7485