A Novel Approach to Reducing Postoperative Intraperitoneal Adhesions Through the Inhibition of Insulinlike Growth Factor I Activity

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Hypothesis: Interference with insulinlike growth factor I (IGF-I) activity, both systemically and intraperitoneally, reduces postoperative intraperitoneal adhesion severity.

Setting: Experimental animal model.

Design, Interventions, and Main Outcome Measures: Adult female rats were subjected to hypophysectomy, sham hypophysectomy (control), IGF binding protein 4 (IGFBP-4) treatment, or albumin treatment (control). All rats underwent laparotomy and uterine horn abrasion with adjacent parietal peritoneal trauma for the purpose of creating postoperative intraperitoneal adhesions. Glucocorticoids and thyroid hormone were replaced in the hypophysectomy group. On postoperative day 10, rats were weighed, subjected to phlebotomy, and killed. Postmortem laparotomies were performed and blinded observers scored uterine-peritoneal adhesions on a 0 to 3 scoring system. Plasma IGFBP-4 levels and organ weights were measured in the IGFBP-4 and albumin treatment groups. Blood samples in all rats were analyzed for IGF-I levels.

Results: Rats with low IGF-I levels (hypophysectomy) and inhibited IGF-I activity (IGFBP-4 treatment) formed significantly less severe adhesions than their control counterparts. As expected, rats in the hypophysectomy group displayed greater weight loss and lower plasma IGF-I levels than sham-treated rats. Rats treated with IGFBP-4 and those treated with albumin demonstrated no differences in body weight, organ weights, IGF-I levels, and IGFBP-4 levels.

Conclusions: Both the reduction of systemic IGF-I levels via hypophysectomy and the inhibition of local intraperitoneal IGF-I activity via IGFBP-4 treatment resulted in diminished postoperative adhesion severity. Treatment with IGFBP-4 may play a role in postoperative adhesion prophylaxis in the future.


Adhesions are abnormal fibrotic connections between body loci that ordinarily have no fixed point of relationship. Although adhesive disease may stem from a number of causes, surgical invasion of body cavities remains one of the foremost. Postoperative intraperitoneal adhesions are particularly troubling to general surgeons and their patients. They cause chronic pain, small-bowel obstruction, infertility, intestinal ischemia, and increased complications on subsequent surgery. Despite numerous diverse approaches for preventing them, intraperitoneal adhesions remain a common condition resulting in enormous costs in terms of patient morbidity, mortality, and medical expense.

Adhesions are the result of the wound healing response, a complex cascade of events that involves an array of cytokines. A multitude of studies have documented the wound healing stimulatory effects of growth factors, including basic fibroblast growth factor, vascular endothelial growth factor, transforming growth factor-β, platelet-derived growth factor, and insulinlike growth factor I (IGF-I).

Insulinlike growth factor I is an anabolic polypeptide that mediates the growth-promoting effects of growth hormone and stimulates a multitude of cellular processes. It has been shown to induce collagen production, angiogenesis, and macrophage recruitment both in vitro and in vivo. This polypeptide is particularly interesting because it is normally 99% bound to IGF-binding proteins (IGFBPs) in the circulation. The family of 6 IGFBPs modulates the activity of IGF-1 by altering the hormone’s ability to bind to its cell membrane receptors. Binding proteins typically inhibit the actions of IGF-1. However, depending on the experimental conditions, IGFBPs may potentiate the ac-
MATERIALS AND METHODS

ANIMAL CARE

All procedures were performed in strict accordance with National Institutes of Health guidelines and the Committee on Animal Research at the University of California, San Francisco. Animals were housed in the animal facility with a controlled environment and a 12-hour light-dark cycle and were acclimatized for 7 days before any procedures. They were allowed water and rat chow ad libitum preoperatively and postoperatively. The animals were inspected daily and were killed if signs of distress were present, according to the guidelines of the Animal Care Welfare Act.

MATERIALS AND REAGENTS

Expression and Purification of IGFBP-4

Escherichia coli BL21 cells (Promega, Madison, Wis) were used to express the IGFBP-4 fusion protein from pGFBP-4, a pET31a expression plasmid (Novagen, Madison, Wis). Cultures (4 × 300 mL) were grown at 37°C on a shaking incubator in LB medium (1% Bacto-tryptone [Difco Laboratories, Detroit, Mich], 0.5% yeast extract, 1% sodium chloride) supplemented with ampicillin, 50 mg/mL. When cultures reached an optical density of 1.0 at 560 nm, isopropyl thiogalactoside was added to a final concentration of 1 mmol/L, and incubation was continued for an additional 5 hours. Cells were harvested by centrifugation (8670 × g, 10 minutes, 4°C), washed, and resuspended in 60 mL of buffer A (50-mmol/L Tris, pH 8.0; 10-mmol/L magnesium chloride; 10-mmol/L imidazole; and 0.1% Triton-X100). The suspension was frozen in acetone–dry ice and thawed in warm water. Lysozyme (1 mg) was added and the freeze-thaw procedure was repeated twice. The resulting mixture was treated with RNase I and DNase I (final concentration of 1 µg/mL) for 20 minutes at 4°C, and phenylmethyl-sulfonyl fluoride was added to the supernatant to a final concentration of 1 mmol/L. The supernatant was then loaded onto a 5-mL nickel-column (Promega). The column was washed with buffer A containing 30-mmol/L imidazole to remove weakly bound material, then bound proteins were eluted with 500-mmol/L imidazole in buffer A. Fractions containing protein were combined (total volume, 20 mL) and immediately dialyzed against 2 L of buffer B (30-mmol/L Tris, pH 8.0) for 1.5 hours. The fusion protein was cleaved by overnight incubation with 2000 U of tobacco etch virus protease at room temperature. The solution was dialyzed against 2 L of buffer B for 1 hour before loading onto the 5-mL nickel-column. The IGFBP-4 was eluted with 40-mmol/L imidazole in buffer B. Fractions containing IGFBP-4 were further purified by reversed-phase high-performance liquid chromatography. Samples were applied to a C-4 column (Vydac, Hesperia, Calif), and the bound material was eluted with a linear gradient of 20% to 40% acetonitrile over 100 minutes at a flow rate of 4 mL/min. All high-performance liquid chromatography solutions contained 0.1% trifluoroacetic acid. Fractions containing IGFBP-4 were frozen in aceton–dry ice and lyophilized. Purified IGFBP-4 could be stored in a desiccator at −20°C for several months.

Other Reagents

Other reagents included levotyroxine sodium (Sigma-Aldrich Corp, St Louis, Mo), phosphate-buffered solution (Mediatech Inc, Herndon, Va), hydrocortisone hemisuccinate (Sigma-Aldrich Corp), and rat albumin (Sigma-Aldrich Corp). Suture material included 6-0 monofilament polypropylene (Davis and Geck, Wayne, NJ) and 4-0 nylon sutures (Davis and Geck).

PROTOCOL FOR HYPOPHYSECTOMY VS CONTROL GROUPS

Fifteen female Fisher rats treated by hypophysectomy (HYP) and 14 subjected to sham hypophysectomy (sham), weighing 200 to 250 g, were obtained (Hilltop Lab Animals, Scottsdale, Pa) and observed for 7 days to confirm the expected lack of weight gain in the HYP group. On day 0, rats were anesthetized with 3% halothane. The animals' abdomens were shaved and prepared with povidone-iodine solution and draped in sterile fashion. In each animal, a 6-cm longitudinal midline incision was made and extended through to the peritoneal cavity with a No. 15 scalpel. The bicornuate uterine horns were visualized. One of the horns was randomly chosen and abraded along 1 cm of its lateral aspect with the use of the broad edge of a No. 15 scalpel. The bicornuate uterine horns were visualized. One of the horns was randomly chosen and abraded along 1 cm of its lateral aspect with the use of the broad edge of a No. 15 scalpel to remove the epithelium. Next, an adjacent 1 × 1-cm window of parietal peritoneum was excised from the abdominal wall with scissors, taking care not to transect any visible vessels. Finally, the uterine serosal surface just distal to the abrasion was approximated to the abdominal wall just distal to the peritoneal window by means of a single 6-0 monofilament polypropylene suture. Bleeding was controlled with direct pressure, and the abdomen was closed in 2 layers with 4-0 nylon sutures. Preoperatively and postoperatively, the water for the HYP

tions of IGF-I. This stimulatory phenomenon has been demonstrated in wound healing with both IGFBP-1 and IGFBP-3.13 Only 1 binding protein, IGFBP-4, is purely inhibitory. It diminishes the effects of IGF-I on many types of connective tissue cells.12-15 Thus, IGFBP-4 represents a convenient pharmacologic means by which excessive fibrosis and healing responses might be modulated in a specific manner.

Given that adhesive disease is a form of overzealous wound healing and that reduction in IGF-I action diminishes the healing response, we questioned whether interference with IGF-I activity both locally and systemically could lessen adhesion formation. We chose to use the rat uterine horn model because it is the best-established animal adhesion model found in the literature.16-22 Specifically, we hypothesized that inhibition of systemic IGF-I production by hypophysectomy would result in less severe postoperative adhesive disease in a rat uterine horn adhesion model. We further hypothesized that local inhibition of IGF-I in normal rats by intraperitoneal IGFBP-4 treatments would similarly reduce adhesion severity.
rats was supplemented with levothyroxine sodium (200 ng/mL) and hydrocortisone hemisuccinate (35 µg/mL). Water consumption was measured every 2 to 3 days, and average daily hormone replacement doses were calculated. On postoperative day 10, rats were reweighed, subjected to phlebotomy, and killed in a carbon dioxide chamber. Postmortem laparotomies were performed, and each intraabdominal 6-0 polypropylene stitch was removed atramatically. Uterine-peritoneal adhesions were scored by consensus of 3 blinded observers according to the following system, modified from Kennedy et al16: 0, no adhesions; 1, thin, filmy adhesions (spontaneously lyse on visualization); 2, moderate adhesions (lyse on traction without tearing of tissue); and 3, severe adhesions (lyse on traction only with tearing of tissue). Disputes were resolved by majority vote. Blood samples were analyzed for serum free thyroxine and plasma IGF-I levels.

PROTOCOL FOR IGFBP-4–TREATED VS CONTROL RATS

Twenty-eight 200- to 250-g healthy female Sprague-Dawley rats (Simonsen Laboratories, Gilroy, Calif) underwent the same abdominal procedure as described earlier. Immediately postoperatively, the rats were randomized equally to either the control group or the treatment group. Twice per day for the next 10 days, the treatment group (IGFBP-4 group) and control animals (albumin group) were briefly anesthetized (3% halothane) and injected intraperitoneally with 3 mL of recombinant rat IGFBP-4 solution (24-µg/mL phosphate-buffered saline) and with 3 mL of rat albumin solution (24-µg/mL phosphate-buffered saline), respectively. On postoperative day 10, rats were reweighed, subjected to phlebotomy, killed, and scored as described earlier. The blood samples were drawn 12 hours after the last intraperitoneal treatment. Organs were harvested and weighed to assess any systemic effects of IGFBP-4 treatments. Small-bowel mucosa weight was measured by scraping and weighing the mucosa from the 50 cm of small bowel distal to the gastroduodenal junction. Blood samples were analyzed for plasma IGFBP-4 and IGF-I levels.

BLOOD CHEMISTRY ANALYSIS

Serum free thyroxine measurements were performed by a microparticle enzyme immunoassay with an analyzer (AxSYM; Abbott Laboratories, Abbott Park, Ill). Plasma IGF-I was measured by radioimmunoassay, as described previously.23 Anti–rat IGF-I antiserum was obtained (Eli Lilly and Co, Indianapolis, Ind).

RADIOIMMUNOASSAY FOR RAT IGFBP-4

The assay buffer contained 50-mmol/L Tris, 50-mmol/L sodium chloride, 0.05% Tween 20, 0.1% EDTA, and 1% bovine serum albumin at a pH of 8.0. Primary antibody (0.2 mL, 1:8000 dilution) was mixed with 0.2 mL of standards (0.34-11.00 ng/mL) or unknowns. After addition of 0.2 mL of IGFBP-4 tracer labeled with iodine 125 (10000–20000 cpm), samples were incubated overnight at 4°C. Bound IGFBP-4 was separated from free IGFBP-4 by double antibody precipitation. This was accomplished by adding 0.1 mL of γ-globulin (1:200), 0.1 mL of secondary goat antirabbit antibody, and 0.7 mL of polyethylene glycol 8000 (9.14%) to the samples. After incubation for 15 minutes at 4°C, samples were centrifuged for 30 minutes at 3000 g. Pellets were counted in a 12-well gamma counter programmed to calculate results (Berthold, Gaithersburg, Md). Serum samples were diluted 1:100 with assay buffer before assay unless otherwise specified.

WESTERN IMMUNOBLOT ANALYSIS

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with 12% polyacrylamide gels to separate proteins. After electrophoresis, proteins were electroblotted to a nitrocellulose membrane at 100 V for 2 hours according to the method of the manufacturer (BioRad, Hercules, Calif). The membrane was dried at 37°C for 5 minutes and washed with 200 mL of 3% NP-40 in Tris buffered saline for 15 minutes. After incubation for 1 hour at room temperature with 1% bovine serum albumin in TBS, the blocked membrane was washed with 0.1% Tween 20 for 15 minutes. The membrane was incubated with 10 mL of antisera (1:10000 dilution) in TBS plus 1% bovine serum albumin and 0.1% Tween 20 for 2 hours, washed with TBS plus 0.1% Tween 20, incubated for 30 minutes with secondary antibody (3.3 mL of goat antirabbit horsradish peroxidase in 10 mL of TBS plus 1% bovine serum albumin plus 0.1% Tween 20) followed by additional washing steps. Finally, the antigen-antibody reactions were visualized with HRP Color Reagent (BioRad) according to the manufacturer’s instructions (BioRad).

STATISTICAL ANALYSIS

All statistical analysis was performed with the Statview 5.0.1 software package (SAS Institute Inc, Cary, NC). The nonparametric adhesion score data were analyzed for differences with the Mann-Whitney U test. Weights, IGF-I levels, free thyroxine levels, and IGFBP-4 levels were analyzed with the 2-tailed t test. Results of parametric data are reported as means ± SDs. P < .05 was considered statistically significant.

RESULTS

HYP VS SHAM GROUPS

All animals survived the surgery and the postoperative period. One rat in the sham group was excluded intraoperatively because of a break in procedure. The adhesion scores in the HYP group were significantly lower than those in the sham group (P < .002) (Figure 1). Rats in the sham group tended to have thicker, more tenacious fibrous tissue between the uterine serosal surface and the peritoneal window than the HYP group. When divided by traction, these adhesions often tore, leaving behind remnants adherent to the abdominal wall. The sham group also more frequently exhibited adhesions to the laparotomy suture line, although quantitation of this tissue was not part of the study design.

As expected, the animals in the HYP group lost significantly more weight than those in the sham group by postoperative day 10 (−25.9 ± 9.3 g vs +2.1 ± 12.5 g; P < .001), confirming successful hypophysectomy. Mean
As shown in Figure 1, adhesion scores in the hypophysectomy (HYP) and sham surgery (Sham) groups. As judged by 3 blinded observers, rats in the HYP group received significantly lower intraperitoneal adhesion scores than the sham-treated counterparts on the 10th postoperative day ($P<.002$).

Figure 2. Adhesion scores in the groups treated with insulinlike growth factor binding protein 4 (IGFBP-4) or albumin. Rats treated twice per day with intraperitoneal IGFBP-4 formed less severe postoperative intraperitoneal adhesions than did the control animals treated with albumin ($P=.02$).

IGFBP-4 concentration in the IGFBP-4–treated rats varied from that of the control animals (albumin). Therefore, it is unlikely that systemic levels of binding protein accumulated or that IGF-I was depleted from the circulation.

Figure 3. Plasma levels of insulinlike growth factor I (IGF-I) and insulinlike growth factor binding protein 4 (IGFBP-4) in IGFBP-4–treated and albumin–treated rats. Neither the plasma IGF-I concentration nor the plasma IGFBP-4 concentration in the IGFBP-4–treated rats varied from that of the control animals (albumin). Therefore, it is unlikely that systemic levels of binding protein accumulated or that IGF-I was depleted from the circulation.

The results from the present study demonstrate that intraperitoneal scarring can be lessened by either systemic reduction in IGF-I production by hypophysectomy or local (intraperitoneal) nullification of IGF-I activity by the application of the purely inhibitory binding protein IGFBP-4. To our knowledge, this investigation is the first to apply the concept of selective inhibition of IGF-I to reduce intraperitoneal adhesion formation.

COMMENT

Numerous approaches to preventing adhesions have been tried, with variable success. Beneficial practices include gentle, atraumatic surgery; avoidance of powdered gloves; and meticulous anastomoses that minimize leakage and infection. Despite intraperitoneal treatment with IGFBP-4, plasma levels of the binding protein were not elevated in the IGFBP-4 group (IGFBP-4 group level, 264.1±56.0 ng/mL; Sham group level, 274.5±58.7 ng/mL). Furthermore, plasma IGF-I concentrations were similar between the 2 groups, with levels in the IGFBP-4 group and albumin group measuring 888.0±81.8 and 907.7±72.1 ng/mL, respectively (Figure 3). These plasma concentrations represent levels at 12 hours after treatment. Total body weight, study period weight change, and organ weights, including small-bowel mucosa, were all similar between the 2 groups (Figure 4). Treatment with IGFBP-4 did not result in statistically significantly lower total body weight or organ weights.
In the HYP vs sham experiment, all of the sham rats received adhesion scores of 2 or 3, while almost half of the HYP rats received scores of 0 or 1, representing minimal or no adhesions. Furthermore, only 1 HYP rat, as compared with about one half of the sham rats, developed the most severe type of scarring, adhesions that could be divided only with tearing of tissue. This difference is clinically important. Animals with adhesion scores of 3 represent the situations with the greatest likelihood of serious intraoperative complication. Therefore, lessening adhesion severity by only 1 point on the scoring scale may correspond to a significant clinical achievement.

The HYP rats were all confirmed to have had complete hypophysectomies on the basis of the total body weight observations and on the day 10 plasma IGF-I concentrations. The HYP group mean weight was one-third less than that of the sham group, and all of the individual HYP rat weights were tightly clustered without any outliers. Similarly, the HYP rats all had plasma IGF-I levels that were an order of magnitude less than those of the sham rats. Because hypophysectomy creates a global hormonal imbalance, including hypothyroidism and adrenal insufficiency, thyroid hormone and glucocorticoids were replaced with levothyroxine and hydrocortisone, respectively. Free thyroxine levels were almost identical between the 2 groups, making it unlikely that the adhesion score differences were related to discrepancies in thyroid state. Our classic endocrine study design prohibited comparison of the glucocorticoid levels between the 2 groups, as the dominant natural glucocorticoid in rats is corticosterone, while corticosteroid replacement in the HYP group was with a standard dose of hydrocortisone. Thus, the HYP rats may have been over-replaced with corticosteroids, a condition that might diminish the wound healing response. This prospect is unlikely, because this dose of hydrocortisone used previously did not result in a healing defect (R. Mueller, MD, oral communication, 1994). Finally, the HYP rats were, by design, underweight compared with their sham counterparts. This difference might have affected the adhesion scores, as it is well known that malnutrition is associated with poor wound healing. However, growth hormone deficiency (the cause of lack of weight gain in HYP rats) results in dwarfism rather than malnutrition.

Animals stripped of their growth hormone responses attain a smaller stature than normal (and therefore less body weight), but are not nutritionally deprived.

In the IGFBP-4 vs albumin experiment, less than one third of the IGFBP-4–treated rats and more than two thirds of the albumin-treated rats received adhesion scores of 3. This second experiment served 2 purposes. First, it was designed to support the results of the HYP vs sham experiment. Indeed, the experiments produced comparable findings. Second, the IGFBP-4 vs albumin experiment demonstrates that local intraperitoneal inhibition of IGF-I activity is feasible.

The IGFBP-4 treatment produced no detectable systemic effects. None of the rats manifested signs of illness as a result of the IGFBP-4 treatments. Neither the total body weights nor the organ weights differed between the IGFBP-4– and albumin-treated rats, indicating that local application of IGFBP-4 did not reduce IGF-I action systemically. Even the small-bowel mucosa weight, a highly sensitive indicator of systemic IGF-I activity, was not depressed in the IGFBP-4–treated rats. Further support of this point is given by the essentially identical IGFBP-4 plasma levels in the 2 groups on day 10, in agreement with other studies. While the half-life of IGFBP-4 is significantly shorter than the 12-hour period between final IGFBP-4 treatment and plasma level measurement, binding protein levels would have been elevated if the protein had accumulated during the 10-day treatment period. Also, we might expect plasma IGF-I concentration to be reduced if IGFBP-4 treatments caused significant systemic IGF-I binding, as IGFBP-4 would essentially “steal” IGF-I from the large, circulation-bound IGFBP-3–acid labile subunit complex, allowing IGF-I to diffuse into tissue and to be excreted. This phenomenon did not occur.

Although the IGFBP-4 group results are not as dramatic as those of the HYP rats, they are still both statistically and potentially clinically significant. The discrepancy, however, might be due to incomplete blocking of IGF-I locally. Thus, the animals in the HYP group may have had less intraperitoneal IGF-I activity than the IGFBP-4–treated rats because of incomplete inhibition in the latter group. Because IGFBP-4 did not cause systemic effects, IGFBP-4 treatment doses may be in-

![Figure 4. Organ and total body weights in rats treated with insulinlike growth factor binding protein 4 (IGFBP-4) or albumin. A. Organ weights in the rats treated with IGFBP-4 were almost identical to those of the control (albumin-treated) rats, suggesting a lack of systemic insulinlike growth factor I inactivation. SB indicates small-bowel. B. The 2 groups also had similar total body weights and weight changes during the 10-day study period, unlike the large disparity between the weights in the hypophysectomy and sham surgery groups.]
creased to achieve greater local IGF-I inhibition and adhesive disease reduction without systemic ramifications.

Although the mechanism by which IGFBP-4 inhibits the activity of IGF-1 was not explored in this study, past investigations suggest that IGFBP-4 competes with IGF-I cell membrane receptors for IGF-I binding.8,15 The affinity of IGFBP-4 for IGF-I is an order of magnitude greater than that of type I IGF receptors.4,43 Miyakoshi and colleagues12 reported that local treatment with intact recombinant IGFBP-4 abrogated IGF-I-associated bone proliferation, while treatment with IGFBP-4 fragment (with 50- to 100-fold less IGF-I affinity) did not, supporting the hypothesis that IGF-I becomes inactive by preferentially binding to IGFBP-4. Therefore, it is likely that, in the present study, IGFBP-4 lessened the severity of postoperative adhesions by binding tightly to IGF-I and preventing its interaction with signaling membrane IGF-I receptors.

Intraperitoneal application of IGFBP-4 to reduce adhesive disease holds several advantages. It can be applied to the entirety of the abdominal contents, achieving a high concentration along the surfaces of structures. At the same time, any IGFBP-4 absorbed into the circulation is rapidly diluted and degraded so that vascularized tissue (ie, perianastomotic tissue) may be exposed to only a small fraction of the concentration present within the abdominal cavity, thereby protecting critical areas. Further studies in this and other animal models are needed to verify the results attained in the present investigation. Also, IGFBP-4 dose-response experiments and more convenient formulations of IGFBP-4 will be required to establish the safest, most effective treatment to reduce postoperative adhesion formation.

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REFERENCES


**Surgical Anatomy**

The Rokitansky-Aschoff sinuses are branching evaginations from the lumen of the gallbladder into the mucosa and muscularis. They play a part in acute cholecystitis and gangrene of the gallbladder.