Heparin binding domain of insulin-like growth factor binding protein-5 stimulates mesangial cell migration

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Heparin binding domain of insulin-like growth factor binding protein-5 stimulates mesangial cell migration. Am. J. Physiol. 273 (Renal Physiol. 42): F899–F906, 1997.—Insulin-like growth factor I (IGF-I) binding protein-5 (IGFBP-5) is produced by mesangial cells (MCs) and likely functions to modulate glomerular IGF-I activity. Although IGFBP-5 may be inhibitory for IGF-stimulated MC activity, preliminary studies suggested that IGFBP-5 acts directly on MCs. To investigate this further, we evaluated the effects of IGFBP-5 on rat MC migration. We found that the carboxy-truncated fragment, IGFBP-5-(1–169), inhibited IGF-I-stimulated migration, but intact IGFBP-5 simulated migration when IGF-I was not present. Demonstration that 125I-labeled IGFBP-5 directly binds to MCs further supports an independent role for IGFBP-5. Because heparin inhibited MC binding of 125I-IGFBP-5, we tested the heparin binding peptide, IGFBP-5-(201–218), for stimulatory activity. IGFBP-5-(201–218) stimulated MC migration, and this effect was inhibited by heparin. Because the disintegrin, kistrin, blocked IGF-I-induced migration but not migration induced by IGFBP-5-(201–218), the migratory induction mechanism for the two peptides is different. These data indicate that separate, specific regions of IGFBP-5 are responsible for interactive effects with IGF-I as well as direct effects on MC activity.

insulin-like growth factor I; glomerulus; chemotaxis; rat

CELL MIGRATION IS CRITICAL for normal development and wound healing (41), and recent studies have elucidated some of the mechanisms required for cells to crawl (13, 53). Cell migration is dependent upon successive formation and release of cell-matrix contacts through interactions with integrins (23, 24, 33). As these focal adhesions reorganize, the underlying cytoskeleton rearranges and shuffles intracellular organelles (14, 30, 54). During this process, endocytosis is greatly increased at the receding side of the cell, and the internalized membrane is inserted into the protruding end (29).

Although cell-matrix interactions are required for migration, a number of growth factors have been shown to stimulate cells to move by activating their respective receptors and stimulating rac and rho (39, 47, 49). The activated receptor subsequently associates with an intracellular integrin domain to cause “inside-out” signaling and activation of the integrin for binding to its matrix ligand (35, 51). Insulin-like growth factor I (IGF-I) has been shown to induce cell migration by this mechanism (48), and we recently reported that IGF-I induces cytoskeletal reorganization in rat glomerular mesangial cells (MC), suggesting that IGF-I may also stimulate MC migration (15). IGF binding protein-5 (IGFBP-5), a secretory product of MC (28), is known to modulate the effects of IGF-I, including IGF-I stimulation of MC proliferation. Based on these studies, we expected that IGFBP-5 would inhibit IGF-I-stimulated MC migration. However, preliminary studies suggested that IGFBP-5 may have direct effects on MC activity.

In the present study, we investigated the mechanisms of IGFBP-5 modulation of IGF-I-stimulated MC migration. We show that the NH2 terminus of IGFBP-5 inhibits IGF-I-induced migration, whereas specific carboxy-terminal residues of IGFBP-5 stimulate MC migration by a mechanism that is independent of IGF-I.

MATERIALS AND METHODS

Materials. Human recombinant IGF-I was purchased from Collaborative Research (Waltham, MA). Intact, human recombinant IGFBP-5 and carboxy-truncated IGFBP-5-(1–169) were purified by IGF-affinity chromatography and reversed-phase high-performance liquid chromatography (HPLC) (6) (provided by Chiron, Emeryville, CA). The IGFBP-5 peptides (130–143, EAVKKDRKKRLTQS; 138–152, KKLQSFKGVGGAENT; and 201–218, RKFYKRPKCPPSRGRKR) were synthesized by solid-phase methodology and purified by HPLC at the Fred Hutchinson Cancer Research Center, Seattle, WA. Kistrin and heparin were purchased from Sigma Chemical, St. Louis, MO. A monoclonal mouse antibody to human IGF-I was obtained from Austral Biologicals, San Ramon, CA.

MC culture. Rat glomerular MC were propagated in culture without supplemental insulin and cloned and characterized as described previously (2, 3). MC were propagated in RPMI 1640 medium containing 20% fetal calf serum (FCS). MC between passages 8–12 were plated in 60-mm dishes at the desired concentration and growth arrested for 48 h by reducing the serum concentration to 2%. At the end of 48 h, the cultures were rinsed, and the medium was changed to the experimental conditions defined below. MC viability is variable in medium containing less than 2% FCS. Proliferation is inhibited and viability is maintained in 2% FCS; therefore, all experiments were conducted in medium containing 2% FCS.

Proliferation assay. Prior to plating MC, a sterile adhesive vinyl strip with perforations 750 µm in diameter (Band-Aid) was applied to one area of the plate. Just prior to the addition of experimental medium and at the termination of the migration experiment, cells were counted in a minimum of 5 perforated areas. The mean number of cells was compared before and after the experiment in each dish. The change in cell number was determined and calculated as a percent of control for each of the experimental conditions.

Migration assay. MC (4 × 105 cells in 4 ml) were plated in a 60-mm dish as described above. In the dish adjacent to the area of the perforated vinyl strip used for cell counting, the cultures were wounded with a sharp razor blade as previously described (34, 43, 44) and rinsed twice with fresh medium. The areas at the wound edge were immediately analyzed to determine an area where the wound was continuous and the
denuded areas were clear of cells. One-millimeter regions of each wound were preselected and marked on the slide before initiation of migration. Experimental medium was added, and cultures were incubated for 48 h, rinsed twice, fixed with 3% toluidine blue. Using a 1-mm square graded eyepiece grid in the microscope, we counted the number of cells migrating across the preselected 1-mm length of the wound in 0.1-mm incremental distances from the wound. For each sample, five separate areas were examined. A minimum of five replicates were examined for each experimental condition. Each experiment was repeated two to three times on separate occasions. Data were analyzed as the total number of migrating cells, with a score based on cell number and distance migrated. The method of analysis did not alter the interpretation; thus, for ease of comparison between experimental conditions, the total number of migrating cells has been calculated and plotted as a percent of control.

Chemotaxis assay. Chemotaxis (concentration gradient-dependent migration) was measured using Nunc Tissue Culture Inserts (Naperville, IL) with 8 µm polycarbonate filters (37). MC were plated on one side of the filter, and IGF-I (100 nM) or IGFBP-5 (201–218) (30 µg/ml) was placed on the other side. At the end of 4 h, cells were scraped off the top side of the filter. The cells that had migrated through the filter were determined by counting 10 fields at ×250 magnification. Five replicates were performed for each condition, and the results were expressed as a percentage of control.

IGFBP-5 receptor binding. To determine IGFBP-5 receptor expression by MC, specific binding of 125I-labeled IGFBP-5 was examined as previously described (4). Confluent monolayers of MC were incubated in serum-free medium overnight. The cells were washed with phosphate-buffered saline (PBS) and incubated with 125I-labeled intact IGFBP-5 in assay buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.1 mg/ml bovine serum albumin) without or with unlabeled IGFBP-5 or heparin for 2 h at 4°C. At the end of the incubation period, the cells were rinsed with PBS and solubilized in 1 N NaOH. Radioactivity of the cell lysates was determined. Specific binding was determined using 300 nM of unlabeled IGFBP-5.

Experimental design. At the time of wounding, the culture medium was changed to include the desired experimental additives. Forty-eight hours later, cells were counted for proliferation and migration. Test substances were added to RPMI 1640 containing 2% FCS. Test substances that were added alone or in combination included IGF-I (0–100 nM), intact IGFBP-5 (0–100 nM), IGFBP-5-(1–169) (30 nM), IGFBP-5 peptides [amino acids 130–143, 138–152, or 201–218 (30 µg/ml)], normal immunoglobulin G, or antibody to IGF-I (0.75 µg/ml), heparin (10 µg/ml), and kistrin (100 nM).

Statistical analysis. Group means were compared by analysis of variance (ANOVA). All results are means ± SE.

RESULTS

IGF-I stimulated MC migration in a dose-responsive manner. An IGF-I concentration as low as 1 nM stimulated MC migration 160% of control (P < 0.01), whereas 100 nM IGF-I stimulated MC migration 200–280% of control values (P < 0.01). When 100 nM IGF-I was incubated with 30 nM intact IGFBP-5, there was a 33% inhibition of MC migration compared with IGF-I alone (Fig. 1). Surprisingly, IGFBP-5 stimulated MC migration when IGF-I was not included during the incubation. To investigate this further, MC migration was evaluated in response to increasing concentrations of IGFBP-5. As shown in Fig. 2, a minimum concentration of 30 nM IGFBP-5 was required to induce a significant response under the conditions of these experiments. Visual inspection of the migrating cells (Fig. 3) revealed marked phenotypic differences depending on the polypeptide. MC stimulated by IGF-I (Fig. 3B) became more bipolar and elongated than untreated controls, whereas the IGFBP-5-stimulated MC (Fig. 3C) displayed an increased number of concentric projections.
and arborizations that were not present in the IGF-I-treated cells. Interestingly, IGFBP-5 treatment resulted in reorganization of the entire colony of cells in addition to migration across the wound in the monolayer, which was not observed in the untreated or IGF-I-treated cells (Fig. 3). IGFBP-5-treated MC migrated further than IGF-I-treated MC, and they appeared to migrate in all directions.

Because these observations suggested that IGFBP-5 has a direct effect on MC function, we examined whether MC could bind IGFBP-5. $^{125}$I-IGFBP-5 was found to specifically bind to MC monolayers with maximum specific binding occurring at 7.5% of total $^{125}$I-IGFBP-5 added (2.5 fmol/10⁶ cells). As shown in Fig. 4A, competition binding studies demonstrate half-maximal inhibition of $^{125}$I-IGFBP-5 binding at 1,000 ng/ml (~31 nM), which is similar to its binding characteristics in cultured osteoblasts (4). To further assess the mechanism of IGFBP-5 binding, we performed competition binding studies with heparin (Fig. 4B) and

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**Fig. 3.** Phenotypic changes of migrating cells. A: control cultures. B: cultures with IGF-I (100 nM). C: cultures with intact IGFBP-5 (30 nM). Note cells migrating across the wound line. The bipolar, stretched phenotype exhibited by MC treated with IGF-I differs from the more compact morphology of untreated cells and the arborized morphology of cells treated with IGFBP-5 (arrows). IGFBP-5-treated cells exhibit a more elongated shape in addition to a spoke-wheel appearance of the perinuclear cytoplasm in cells that are beginning to separate from each other and have not yet moved across the wound.

**Fig. 4.** MC binding of $^{125}$I-labeled intact IGFBP-5. A: competition of $^{125}$I-IGFBP-5 binding by excess unlabeled ligand (intact IGFBP-5). B: competition curve with heparin.
found that heparin was as potent an inhibitor of 

\(^{125}\text{I}-\text{IGFBP-5}\) binding to MC as it is for osteoblasts (4).

This suggested that heparin interfered with the MC-

\text{IGFBP-5}\) interaction by its attachment to the heparin binding domain located within the carboxy-terminal residues 201–218 of IGFBP-5.

Because of this observation, we next examined whether a peptide that contained the heparin binding domain was capable of stimulating MC migration. As shown in Fig. 5, 30 µg/ml IGFBP-5-(201–218) markedly stimulated migration. This effect was specific for the heparin binding domain, since other basic residues within IGFBP-5, such as IGFBP-5-(130–143) and IGFBP-5-(138–152), did not stimulate migration. Moreover, the carboxy-truncated peptide, IGFBP-5-(1–169), had no effect on MC migration. Consistent with the effect of heparin to inhibit IGFBP-5 binding, heparin also inhibited IGFBP-5-(201–218) stimulation of MC migration (Fig. 6). This further supports the notion that this region of IGFBP-5 interacts directly with MC binding sites, as it is responsible for induction of direct effects on MC migration and phenotype.

IGF-I-stimulated migration of vascular smooth muscle cells requires engagement of the vitronectin receptor, \(\alpha_V\beta_3\), which is blocked by kistrin (34). To explore the possibility that the stimulatory effect of IGFBP-5 on MC were mediated through the heparin binding domain of the 201–218 peptide; yet our initial data with combinations of IGFBP-5 and IGF-I had shown that IGFBP-5 could also inhibit IGF-I action. We tested various regions of IGFBP-5 in combination with IGF-I to identify the region of IGFBP-5 responsible for inhibiting IGF-I action of MC. As shown in Fig. 9, IGFBP-5-(1–169), which had no direct effect on MC migration, prevented IGF-I stimulation of MC migration.

These data demonstrated that direct effects of IGFBP-5 on MC were mediated through the heparin binding domain of the 201–218 peptide; yet our initial data with combinations of IGFBP-5 and IGF-I had shown that IGFBP-5 could also inhibit IGF-I action. We tested various regions of IGFBP-5 in combination with IGF-I to identify the region of IGFBP-5 responsible for inhibiting IGF-I action of MC. As shown in Fig. 9, IGFBP-5-(1–169), which had no direct effect on MC migration, prevented IGF-I stimulation of MC migration.
migration, was a more potent inhibitor of IGF-I stimulation than intact IGFBP-5. Moreover, when MC were incubated with both IGFBP-5-(201–218) and IGF-I, the effect on MC migration was additive. This further supports the data described above which show that the heparin binding domain directly stimulates MC by an independent mechanism and it does not inhibit IGF-I action. The intermediate effect of intact IGFBP-5 in the presence of IGF-I suggests that partial inhibition of IGF-I-stimulated migration is the net effect of independent and interactive effects of IGFBP-5.

MC numbers were monitored during the course of the migration assays to assure that the migratory responses were not simply the result of cellular proliferation. The changes in cell numbers for one set of experiments were as follows: control, 100 ± 10%; IGF-I, 111 ± 10%; IGFBP-5, 95 ± 6%; IGFBP-5-(1–169), 96 ± 9%; and IGFBP-5-(201–218), 101 ± 8% (ANOVA, P > 0.05). For all of the experiments described above, cell numbers at the end of 48 h varied ± 10% from controls (ANOVA, P > 0.05).

**DISCUSSION**

The effects of growth factors on proliferation, cell shape, and cell motility are of considerable interest in understanding how cells populate growing organs during development, spread during tumor metastasis, change phenotype, and function during tissue injury and repair (17, 41). In the case of the effects of IGF-I on cells, a family of IGFBPs are known to modulate IGF-I action (31). Members of this family inhibit IGF-I action, shift the cellular response to IGF-I from proliferation to differentiation (31, 48), and in some cases have effects on cells that are independent of IGF-I. We chose to evaluate the effects of IGFBP-5 on MC migration, because MC synthesize IGFBP-5 in culture (28) and in vivo (38, 46), and its expression corresponds with critical stages of nephrogenesis, including differentiation of the MC (38).

The independent effects of IGFBP-5 on MC migration were induced by the heparin binding peptide, IGFBP-5-(201–218). This basic amino acid-rich region of IGFBP-5 was a potent stimulator of MC migration. The effects of IGFBP-5-(201–218) on MC migration appear to be specific, as migration was not stimulated by the carboxytruncated fragment, IGFBP-5-(1–169), nor by two other IGFBP-5 peptides with charges similar to the 201–218 peptide. It is also of note that we demonstrated that MC express a cell surface binding site for IGFBP-5 with binding characteristics similar to those previously described in osteoblasts (4). Heparin inhibition of IGFBP-5 binding to MC strongly suggests that the heparin binding domain in the 201–218 region is an important cell surface binding site for both MC and osteoblasts (4). These data argue that the effects of IGFBP-5 on MC migration are specific and contained within the 201–218 region. Since kistrin did not block the stimulatory effect of either intact IGFBP-5 or IGFBP-5-(201–218), we conclude that kistrin does not bind IGFBP-5 and that the αVβ3 integrin does not mediate the migratory stimulus of IGFBP-5 on MC. Furthermore, because IGF-I and IGFBP-5 treatments of MC were associated with strikingly different changes in MC phenotype and because IGF-I but not IGFBP-5-(201–218) induced chemotaxis, we believe that the mechanisms responsible for migration induced by these two ligands are different.

MC also synthesize IGF-I (19) and express IGF-I receptors (1, 9, 10, 20), and IGF-I induces MC proliferation (1, 10), cytoskeletal reorganization (15), and a change in the composition of extracellular matrix that MC secrete (25, 50). IGF-I plays an important role...
during kidney development (11) and responses to renal injury (26, 36). Because MC synthesize IGF-I and IGFBPs (10, 19, 28), IGF-I receptor activity is under autocrine control. Recently, we demonstrated that IGF-I induces cytoskeletal rearrangements in rat MC typical of migrating cells (15). Thus we expected that, similar to other cells, IGFBP-5 would induce MC migration. Our results confirm that IGF-I induces MC to migrate, and similar to studies in other cells (22), IGF-I is chemotactic for MC, as they migrate in a directional manner toward a concentration gradient of IGF-I. In our studies, IGF-I-stimulated MC migration was inhibited by an antibody to IGF-I, confirming the specificity of IGF-I. In porcine vascular smooth muscle cells, IGF-I-induced migration requires serum, a source of vitronectin, and attachment to the vitronectin receptor, αvβ3 (33, 34). Kistrin, a disintegrin that binds to the vitronectin receptor and blocks cellular attachment to ligands for this receptor, inhibits IGF-I-stimulated migration (34). This suggests that IGF-I-induced migration requires cell-matrix attachment via αvβ3. IGF-I treatment of porcine vascular smooth muscle cells also increases the expression of αvβ3, which may contribute to the enhanced migratory response (34). These findings in vascular smooth muscle cells are relevant to our studies, in which kistrin similarly inhibited IGF-I-induced MC migration. Like vascular smooth muscle cells, MC also express both the αvβ3 and αvβ1 integrins (21, 45, 52).

The interactive effects of IGF-I and IGFBP-5 influence their activity, as binding to each other protects each of these factors from proteolysis (8). IGFBP-5 binds to extracellular matrix, where it can serve as a reservoir of IGF-I (18, 32). When matrix bound, IGFBP-5 has reduced affinity for IGF-I, which may facilitate the release of intact IGF-I for binding and activation of the IGF-I receptor (32). This may explain why in some cases IGFBP-5 potentiates IGF-I-stimulated migration (5, 32). Alternatively, binding of IGF-I to IGFBP-5 may prevent either protein from binding to its respective receptor and thereby blunt its activity. We evaluated the interactive effects of IGF-I and IGFBP-5 with the expectation that IGFBP-5 would blunt the migratory response to IGF-I (27). Although this was confirmed, we found that the inhibitory effects of IGFBP-5 on IGF-I were not as great as anticipated, because of the independent stimulatory effects of IGFBP-5 on the MC. Because MC synthesize both IGF-I (10) and IGFBP-5 (28) and express receptors for each of these ligands (1, 10), we presume that the migration observed under experimental conditions is the net effect of both independent and interactive effects of IGF-I and IGFBP-5. Although the interactive effects of IGFBP-5 and IGF-I have been well established, the data presented here demonstrate that IGFBP-5-(1–169) contains the region responsible for inhibiting IGF-I-induced migration. Interestingly, carboxy-truncated IGFBP-5-(1–169) was a more effective IGF-I-inhibitor than intact IGFBP-5, despite having markedly reduced affinity for IGF-I (4). This surprising finding led to the observations that intact but not carboxy-truncated IGFBP-5 independently stimulated MC migration and that the independent activity of IGFBP-5 resides with the carboxy-terminal region of the molecule.

These data add to a growing body of evidence showing that IGFBPs can affect cell behavior by mechanisms that are independent of their IGF binding activity (4, 5, 12, 40, 42). Bar et al. (12) found that cellular glucose uptake was stimulated by endothelial cell-derived IGFBPs and that peptides corresponding to the heparin-binding domains of IGFBP-3 and IGFBP-6 mimicked this effect (12, 16). Moreover, IGFBP-1 has been shown to stimulate cell migration by binding to the αvβ3 integrin via the RGD sequence (33). Since IGFBP-5 lacks a RGD sequence, it likely stimulates migration by a different mechanism. The heparin binding domain of IGFBP-5 has been implicated in regulating the proteolytic degradation of IGFBP-5 through its binding to glycosaminoglycans (7) and in mediating the binding of intact IGFBP-5 to selected components of fibroblast extracellular matrix where it functions to enhance fibroblast proliferation (32). Our finding that the heparin binding peptide, IGFBP-5-(201–218), is capable of stimulating MC migration is consistent with the notion that this carboxy-terminal region of IGFBP-5 must be available for binding to cell surface receptors either as part of the unbound intact molecule or as a peptide following proteolytic degradation.

The novel mechanisms of MC migration raise important questions regarding IGFBP-5 function in the kidney. In several kidney diseases, mesangial deposition of extracellular matrix is enhanced, and some of these components such as laminin, fibronectin, and collagen IV avidly bind intact IGFBP-5 (43). Thus excess accumulation of extracellular matrix within diseased glomeruli could result in a repository for intact IGFBP-5. The extent of subsequent proteolysis of intact IGFBP-5 to form NH2- and COOH-terminal fragments would be another level of regulation of cell migration. For example, increased proteolytic degradation could result in a shift to more IGFBP-5-(1–169), which would inhibit IGF-I-induced MC migration or proliferation. Alternatively, if proteolysis was more selective and resulted in an increased IGFBP-5-(201–218) concentration, then MC movement could be enhanced, regardless of the degree of IGF-I receptor activation, and, in fact, might lead to additive effects as we observed in vitro. The concentration of glycosaminoglycans within extracellular matrix would be another consideration that could impact MC migration, since we know that heparin inhibits the migratory response to IGFBP-5-(201–218). This finding might explain earlier results showing that heparin inhibits MC migration (44) and may lead to therapeutic uses of glycosaminoglycans to selectively inhibit excessive MC migration.

In summary, we have shown that IGFBP-5 specifically binds to and stimulates MC migration and changes its phenotype. The region of IGFBP-5 that is responsible for the direct effects on the MC resides within the carboxy-terminal residues, 201–218, which likely mediate binding of IGFBP-5 to its cell surface receptor.
These residues also contain the heparin binding domain, which explains the capacity of heparin to inhibit IGFBP-5-mediated MC migration. Similar to other molecules, these findings have important implications in understanding mechanisms of MC migration during glomerulogenesis and in glomerular diseases such as membrandroproliferative glomerulonephritis, where MC migration into the subendothelial space is a characteristic finding.

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