Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) Inhibits Breast Cancer Cell Motility

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ABSTRACT

The breast cancer malignant phenotype is regulated by steroid hormones and peptide growth factors. We have shown previously that insulin-like growth factor-I (IGF-I) stimulates cell motility in a metastatic cell line, MDA-231BO. In this study, we show that neutralization of IGF action by a type I IGF receptor (IGFR1) blocking antibody or neutralization of IGF-I by IGFBP-1 reduced cell motility. However, in addition to inhibiting IGF effects, IGFBP-1 also diminished basal motility. Because IGFBP-1 contains a RGD motif important in binding of fibronectin to its α5β1 integrin receptor, we examined the effect of inhibiting integrin function on cell motility. As expected, disruption of fibronectin-integrin interactions interrupted basal motility in MDA-231BO cells. In addition, disruption of integrin function by an α5β1 blocking peptide also inhibited IGF stimulation of cell motility. To determine whether integrin function could interfere with IGF signaling, we used an α5β1 blocking peptide to show that in MDA-231BO cells integrin occupancy appeared necessary for phosphorylation of insulin receptor substrate-2 but not for IGFR1 activation. We conclude that IGFR1 and integrin action are linked in these breast cancer cells as disruption of integrin binding to its receptor influences IGF signaling pathways. Moreover, IGFBP-1 could have dual effects on cancer cell motility by disrupting both receptor systems.

INTRODUCTION

Abundant evidence suggests that the IGFs participate in maintenance of the malignant phenotype. In several cancer model systems, the IGFs enhance proliferation, protect cells from apoptosis, and stimulate several aspects of the malignant phenotype (1). Overexpression of IGF-I in the mammary gland enhances tumorigenesis in mice deficient in the p53 gene (2). Moreover, high serum levels of IGF-I have been associated with an increased risk of breast, prostate, and colon cancer (3). If the IGFs are important in cancer cell biology, then inhibition of IGF function should be a reasonable anticancer strategy.

Both of the IGF ligands, IGF-I and IGF-II, interact with specific cell surface receptors to stimulate intracellular signaling pathways (4). It has been shown that IGF-I and IGF-II can interact with IR, IGFR1, and hybrid IR/IGFR1 to demonstrate that multiple receptors play roles in determining IGF action. Both IR and IGFR1 are transmembrane tyrosine kinase receptors. In theory, a small molecule inhibitor could be developed to inhibit the kinase activity of the IGFR1. However, because both IR and IGFR1 are highly homologous in the tyrosine kinase domain, efforts thus far have not yielded a selective inhibitor (5, 6). Moreover, given the importance of IR action in peripheral normal tissues (7), it would then be necessary to deliver the IR and IGFR1 tyrosine kinase inhibitor directly to breast cancer cells. At present, existence of such a specifically targeted delivery system seems unlikely.

Unlike other tyrosine kinase receptors involved in malignancy, activation of IGFR1 by overexpression alone is not seen in cells. In transfection model systems, ligand binding to IGFR1 is required to initiate signal transduction (8). Whereas src transformed cells can activate IGFR1 in cell culture models, this pathway has not been shown to occur in human tissue specimens (9). Thus, another way to block IGF action would be to disrupt ligand/receptor interaction. Indeed, monoclonal antibodies that inhibit IGF binding to IGFR1 have been shown to be useful in animal models of breast cancer (10, 11).

Another anti-IGF strategy involves ligand neutralization by providing a “target decoy” for IGF-I and IGF-II. Dunn et al. (12) have shown that the extracellular ligand binding domain of IGFR1 inhibits metastasis in an animal model. IGF action is controlled by high affinity binding proteins. To date, six distinct IGFBP species have been cloned, although there are proteins with shared domains, and have been called IGFBP-related proteins (13, 14). Because the IGFBPs have higher affinity for IGF-I and IGF-II than the receptors, it would also be possible to neutralize IGF action with these naturally occurring proteins. We have shown that inhibition of IGFR1 function by an excess of IGFBP-1 inhibits monolayer growth of MCF-7 cells (15, 16). In addition, conjugation of IGFBP-1 with polyethylene glycol is effective in inhibiting tumor growth in athymic mouse models (17). Our data show that in this breast cancer cell line, IGF-I stimulates cell proliferation and inhibits apoptosis. Thus, ligand neutralization of IGF-I by IGFBP-1 blocks activation of the IGFR1 to impact tumor cell growth.

We and others have shown recently that IGFR1 activation in some breast cancer cell lines does not enhance proliferation (18, 19). In contrast, we have shown that IGFR1 activation augments cell adhesion and motility in two cell lines derived from metastatic deposits in vivo (LCC6 and MDA-231BO). In these cells, we found that IGFR1 activation of IRS-2 was responsible for these effects. In this study, we wanted to determine whether rhBP-1 would also function to inhibit IGF-mediated adhesion and cell motility. As expected, we found that rhBP-1 could neutralize IGF action in these cell lines. However, we noticed that rhBP-1 by itself had inhibitory effects on cell motility. Because rhBP-1 contains an arg-gly-asp (RGD) motif important in integrin binding (20), it was possible that rhBP-1 had dual effects on both integrin and IGFR1 function. We found that this is indeed the case; in fact, activation of appropriate signaling pathways downstream of IGFR1 requires ligation of α5β1 integrin.

MATERIALS AND METHODS

Cells and Reagents. MDA-MB-231 and the MDA-231BO variant were kindly provided by Dr. Toshiyuki Yoneda (University of Texas Health Science Center at San Antonio, San Antonio, TX). The MDA-231BO is a bone-seeking metastatic variant of MDA-MB-231 (21). MCF-7L was from C. Kent Osborne (Baylor College of Medicine, Houston, TX). All of the chemicals and reagents were purchased from Sigma (St. Louis, MO) unless noted otherwise. The culture medium and FN were from Life Technologies, Inc. (Rockville, MD). IGF-I was purchased from Gro Pep (Adelaide, Australia). rhBP-1 was purified as described previously (15). RGD and RGE synthetic peptides (GRGDTP and RGES) were from Sigma. Horseradish peroxidase-conjugated RC-20 antiphaso-
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IGF-I Stimulation of MDA-231BO Migration Is Mediated through IGFRI. MDA-231BO and MDA-MB-231 cells were pretreated with a monoclonal antibody (αIR3) that inhibits IGF-I activation of IGFRI (10, 23). As shown in Fig. 1, IGF-I-stimulated migration was completely inhibited in the MDA-231BO cells, whereas MDA-MB-231 cells were unaffected by either IGF-I or αIR3. To examine the effect of αIR3 on activation of downstream signaling pathways, we treated cells with IGF-I with and without the antibody. Total antiphosphotyrosine immunoblotting demonstrated that IGF-I-stimulated phosphorylation of IRS species was inhibited by αIR3 (Fig. 2) in all of the cell lines.

Basal and IGF-I-stimulated Migration Are Inhibited by rhBP-1. We have shown that IGF-I action in MCF-7 cells can be inhibited by an 8-fold molar excess of rhBP-1 (15). To determine whether rhBP-1 could also neutralize IGF-I-mediated cell motility, we pretreated cells with 40 μM rhBP-1 before exposing cells to 5 nM IGF-I. rhBP-1 inhibited stimulation of cell motility by IGF-I in MDA-MB-231BO cells and did not affect the parent cell line (Fig. 3). In contrast to the results with αIR3, rhBP-1 inhibited the basal levels of migration. Thus, both αIR3 and rhBP-1 clearly inhibited IGF-I effects by blocking the ability of IGF-I to activate IGFRI. However, rhBP-1, and not αIR3, inhibited motility even in the absence of IGF-I. These data suggested that rhBP-1 could influence additional aspects of cell motility that are independent of IGFRI function. Because rhBP-1 contains an integrin binding motif (20), we examined the possibility that interruption of integrin function by rhBP-1 could account for this inhibition of basal motility.

Basal and IGF-I-stimulated Migration Is Inhibited by a Synthetic RGD Peptide and α5 Integrin Antibody. To migrate, cells must establish dynamic and highly regulated adhesive interactions with the extracellular matrix. In our assays, the serum-free defined medium contains FN, a component of extracellular matrix. Its major receptor on the epithelial cell surface is α5β1 integrin. Because rhBP-1 has been shown to bind α5β1 integrin (20) via its RGD domain, it was possible that rhBP-1 was influencing FN interactions with its integrin receptor to affect cell migration. To test this possibility, we used a synthetic RGD containing peptide (GRGDTP), which blocks FN binding to its receptor (24). As shown in Fig. 4, 25 μg/ml pretreatment of GRGDTP greatly diminished both basal and IGF-I-induced cell migration in MDA-231BO but not in MDA-MB-231. The control RGE peptide had no effect on cell migration up to the concentration of 100 μg/ml. Whereas it was expected that the RGD peptide would inhibit basal migration, it was surprising that this peptide also inhibited IGF stimulated motility. This result suggests that inhibition of cell migration by rhBP-1 may be because of dual effects, neutralization of IGF-I, and interaction with the integrin system. However, integrin function may be less important in mediating IGF-I-stimulated proliferation, as the RGD peptide did not affect the ability of IGF-I to stimulate cell growth in MCF-7 (data not shown).

To determine whether α5β1 integrin was a relevant target for rhBP-1, we measured expression of this integrin in MDA-231, MDA-231BO, and MCF-7 cells. Fig. 5 shows that both α5 and β1 integrin were expressed in MDA-231BO and MDA-MB-231 cells, whereas in MCF-7L, β1 integrin expression was low and α5 integrin was almost mediated through IGFRI.
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Fig. 1. IGF-I-induced cell motility is inhibited by blockade of IGFR1. Cells were preincubated with the IGFR1 blocking antibody αIR3 then treated in the absence (□) or presence of 5 μM IGF-I (■). Migration in a modified Boyden chamber was assayed. IGF-I (5 μM) stimulated the cell migration in MDA-231BO (left panel) and was inhibited by αIR3 (1 μg/ml). Motility of MDA-MB-231 cells was not affected by IGF-I or αIR3 (right panel); bars, ± SD.

Fig. 2. Phosphorylation of IRS-2 is inhibited by αIR3. Breast cancer cell lines were incubated in SFM and treated with nothing, IGF-I (5 μM), and αIR3 (1 μg/ml) plus IGF-I for 10 min. IRS-2 (in MDA-231BO and MDA-MB-231) and IRS-1 (in MCF-7L) activation was measured by antiphosphotyrosine antibody immunoblotting. αIR3 inhibited phosphorylation in all cell lines.

undetectable. IGF-I treatment had no effect on α5β1 integrin expression. We have shown previously that MCF-7L cells do not migrate in response to IGF-I in our FN containing system (18) suggesting that expression of α5 integrin could be necessary for IGF-mediated motility. To test this directly, we incubated cells with a neutralizing antibody to α5 integrin. Fig. 6 shows that increasing concentrations of the antibody diminished basal and IGF-I-mediated MDA-231BO motility. Thus, inhibition of α5 integrin by an antibody that interferes with FN binding to its integrin receptor diminished the ability of IGF-I to enhance cell motility. Notably, the parent MDA-MB-231 cell line expressed α5 integrin yet did not migrate in response to IGF-I suggesting that integrin expression alone is not sufficient to cause IGF-I-induced motility. However, these data indicate that integrin-mediated cell adhesion may be required in the IGF-I stimulated cell migration as well as in the basal level of migration. Because both RGD peptide and rhBP-1 inhibited IGF effects, these findings raised the possibility that IGFR1 function may be linked to integrin occupancy.

IGF-I-stimulated IRS-2 Phosphorylation in MDA-231BO and MDA-MB-231 Requires Cell Attachment. We next examined whether cell adhesion status affected IGFR1 signaling. Nearly confluent cells were serum-starved overnight. Cells were then detached or left in monolayer culture and stimulated with IGF-I. As shown in Fig. 7, when cells were attached to substrate IGF-I stimulated tyrosine phosphorylation of IRS molecules. As we have shown previously, IRS-1 is stimulated in MCF-7 cells (25), whereas IRS-2 is phosphorylated in the MDA-MB-231 cells (18). However, when cells were detached, MDA-231BO and MDA-MB-231 cells had diminished phosphorylation of IRS-2, whereas MCF-7 still seemed to appropriate phosphorylate IRS-1. Thus, substrate attachment appears to be required for IRS-2 activation in MDA-MB-231 and MDA-MB-231BO cells.

rhBP-1 and Synthetic RGD Peptide Inhibit IRS-2 Phosphorylation in MDA-MB-231BO. Our data suggest that both IGFR1 and integrin need to be ligand occupied for IRS-2 phosphorylation. To additionally explore this possibility, we incubated cells with the RGD peptide or rhBP-1 before IGF-I exposure. Fig. 8 shows that rhBP-1-inhibited IGF-I induced IRS phosphorylation in all of the cell lines examined. In contrast, RGD peptide only inhibited the ability of IGF-I to activate IRS-2 in MDA-231BO cells. IRS phosphorylation in MCF-7 and MDA-MB-231 was not affected by RGD peptide. Control RGE peptide had no effect on IRS phosphorylation (data not shown). Thus, IRS-2 phosphorylation can be inhibited both by inhibiting IGFR1 activation and by interrupting integrin binding to FN. rhBP-1 inhibits IGF action in all of the cell types because of its ability to prevent ligand-receptor interactions. In cells with IGF-I enhanced motility and adhesion, RGD peptide also inhibits effective phosphorylation of the appropriate IRS substrate.

RGD Peptide Does Not Affect IGFR1 Autophosphorylation. Zheng and Clemmons (26) have shown that occupancy of the αVβ3 integrin is required for IGFR1 activation in vascular smooth muscle cells. To determine whether a similar requirement for α5β1 occupancy was required in these breast cancer cells, we examined the ability of RGD peptide to influence IGFR1 autophosphorylation. Cells were treated in the absence or presence of IGF-I and membranes were
isolated. Fig. 9 shows that IGFR1 was detected in the membranes and that treatment with IGF-I stimulated autophosphorylation of the subunit. IGF-I could be neutralized by excess IGFBP-1 as shown previously. However, neither RGE or RGD peptide affected activation of IGFR1. Thus, inhibition of integrin binding to FN does not influence IGFR1 autophosphorylation but likely interrupts signaling pathways downstream of the receptor.

**DISCUSSION**

In addition to the potent mitogenic and antiapoptotic effects on mammalian cells, IGF-I has been reported to stimulate cell migration in breast cancer (27), neuroblastoma (28), smooth muscle (29), and colonic epithelial cells (30). We have also shown that 5 nM IGF-I can stimulate MDA-231BO cell migration (18). In contrast to the report by Doerr and Jones (27), we did not detect IGF-I-induced cell migration in MCF-7L cells or in the parent MDA-MB-231 cells. We also did not find IGF-I to be a chemotactic factor perhaps because of differences in experimental conditions and substrates (Table 1). As expected, inhibition of IGFR1 with the monoclonal antibody IR3 blocked the IGF-I-mediated migration as well as decreased the downstream signaling molecule IRS-2 phosphorylation in MDA-231BO cells (Figs. 1 and 2). IGFBP-1, a neutralizing reagent for IGF-I action, has similar inhibitory effects on MDA-231BO cell migration and IRS-2 phosphorylation (Figs. 3 and 9). These data suggest that IGFBP-1 inhibits breast cancer cell motility.

rhBP-1 binds IGF-I with high affinity and can be used as an inhibitor of IGF-I action (15). In this study, we found that rhBP-1 not only inhibited IGF-I-stimulated IRS-2 phosphorylation and cell migration as mentioned above but also significantly reduced cell migration in the absence of IGF-I. IGFBP-1 contains an RGD integrin recognition sequence and has been reported to bind α5β1 integrin.
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The physiological consequences of an IGFBP-1/α5β1 integrin interaction are not clear. IGFBP-1 has been reported to stimulate cell migration in Chinese hamster ovary and pSMC cells (20, 31). However, IGFBP-1 has also been shown to inhibit cytrophoblast attachment to FN and invasion into decidualized endometrial stromal cultures (32). Thus, it appears that the effect of IGFBP-1 on integrin function could be cell specific. In addition, we used rhBP-1 at concentrations far beyond the physiological range.

There is growing evidence that cell proliferation, differentiation, and migration are regulated by the integrated action of growth factor and integrin signaling events (27, 33–36). Blocking ligand occupancy of the αVβ3 integrin inhibits IGF-I signaling in pSMC (26). Jones et al. (37) have reported that ligand occupancy of the αVβ3 integrin is necessary for SMC to migrate in response to IGF-I. By using the synthetic αVβ3 antagonists, pSMC replication and migration in response to IGF-I were also attenuated (29). α5β1 integrin, a major mediator of cell binding to FN, has been reported to interact with oncogenic SHC proteins, and to regulate breast cancer cell adhesion and motility (38). Thus, a large body of evidence suggests that integrin and IGFR1 signaling pathways interact.

Our data suggested that rhBP-1 inhibited basal and IGF-I-stimulated cell motility. Because our migration assay medium contains FN, it was quite possible that IGFBP-1 competes with FN and blocks integrin-mediated effect required for cell migration. We used a synthetic RGD peptide, which blocks FN binding to its receptor, to indirectly test this hypothesis. Indeed, RGD peptide had the similar inhibitory effect on both basal and IGF-I-induced MDA-231BO cell migration. This result suggested that IGFBP-1 may play dual roles in the cell migration process. IGFBP-1 may neutralize IGF-I action and inhibit of integrin signaling. This effect may be specific for specific substrates and integrins, as we do not detect IGF-I-induced cell motility in type I collagen (18).

Our previous study showed that MCF-7L cells in which IRS-1 was phosphorylated in response to the IGF-I treatment have negligible basal migration under our experimental conditions (18). Whereas in MDA-231BO and MDA-MB-231, the two cell lines that phosphorylate IRS-2 on IGF-I treatment, basal migration was much higher. To determine whether integrin expression accounted for the differences in basal cell migration, we examined the α5β1 levels in these cell lines by immunoblot. As shown in Fig. 5, both integrins were expressed in MDA-231BO and MDA-MB-231 cells at a relatively high level, whereas in MCF-7L, the β1 expression was low and α5 was almost undetectable. The initial event in the migratory cycle is adhesion formation and protrusion at the cell leading edge. Subsequently, the cell must be able to release adhesions at the cell rear (39). Thus, the adhesion proteins, such as FN, and their receptors, such as α5β1 integrin, constitute a versatile recognition system providing cells with anchorage, adhesion-dependent cooperative signals and traction for migration. In accordance with the α5β1 integrin expression profiles in MDA-231BO, MDA-MB-231, and MCF-7L, we had shown previously that MCF-7L had negligible adhesion to FN compared with that of MDA-231BO and MDA-MB-231 (18). Whereas the differential IRS activation may partially explain the different behavior of cell migration in MDA-231BO, MDA-MB-231, and MCF-7L (18), the lack of FN α5β1 integrin receptor may also account for the poor IGF-induced cell migration in MCF-7L cells. Of note is that Doerr and Jones (27) have shown that IGF-I enhances migration and is a chemotactic factor in MCF-7 cells on vitronectin and collagen. Similar to our findings, these authors showed that MCF-7 cells do not migrate on FN, demonstrating that specific integrin species may interact with IGF signaling pathways (18).

It is also clear that the ability of IGFR1 to activate downstream signaling pathways in breast cancer cells depends on the function of the integrin receptors. First, MCF-7L cells detached from a substrate can still phosphorylate IRS-1 in response to IGF-I, whereas IRS-2 phosphorylation in MDA-231 and MDA-231BO cells was diminished after detachment from the substrate. Secondly, inhibition of α5β1 integrin occupancy by RGD peptide diminished IRS-2 phosphorylation in MDA-231BO cells, whereas autophosphorylation of IGFR1 itself was not affected. Thus, in MDA-231BO cells, occupancy of both IGFR1 and α5β1 integrin are required to phosphorylate IRS-2 and initiate cell motility.

The mechanism of preferential metastasis of breast cancer to bone is not understood. MDA-231BO, an exclusive bone-seeking subclone of the human breast cancer cell line MDA-231, has distinct biological characteristics compared with the parental cell line (21). For example, IGF-I and -II markedly promoted the anchorage-independent growth in MDA-231BO, but not in MDA-MB-231, and transforming growth factor β profoundly inhibited the growth of MDA-MB-231 but not of MDA-231BO. We have shown previously that MDA-231BO has increased IRS-2 activation and signaling compared with that of parental cell line MDA-MB-231 (18). During the selection for bone metastasis, MDA-231BO cells became less adherent to FN while becoming more responsive to IGF-I. Despite lower basal migration compared with that of MDA-MB-231, MDA-231BO cell migration was stimulated by IGF-I, and MDA-MB-231 cell migration was not. Whereas our experiments using an antisense strategy to down-regulate...
IRS-2 expression show that activation of IRS-2 is required for IGF-induced mitotony, the molecular mechanisms for these observations have not been fully explained. We have shown that the MDA-231BO cell line has a slightly higher level of IGFR1 compared with the parent cell (18). However, this finding alone cannot account for the difference in the ability of integrin occupancy to affect IRS-2 phosphorylation or the observed difference in basal adhesion to FN. It is possible that cellular localization of IGFR1 and integrin receptors differ between the two cell lines. It is also possible that other integrin receptors vary in their level of expression.

Breast cancer metastasis requires a complex cascade of events that involve multiple molecular mechanisms. Interestingly, both integrin and IGF receptors are functionally linked in this process. Inhibition of either or both receptors affects the ability of MDA-231BO cells to respond to IGF-I. rhBP-1 is a more potent inhibitor of cell motility than α6β3, an antibody that specifically inhibits IGFR1. Whereas α6β3 inhibits the ability of IGF-I to stimulate mitotony, rhBP-1 blocks both basal and IGF-induced mototony. The dual function of rhBP-1 in both IGFR1 and integrin action could be exploited to understand how these pathways intersect to influence breast cancer cell metastasis.

As shown by several laboratories, IGFR1 activation participates in all of the aspects of the malignant phenotype (12, 25, 40, 41). In MDA-231 cells, IGFR1 appears to enhance cell motility but, even when overexpressed, does not influence cell proliferation or survival (42). Thus, IGFR1 has defined functions in specific cells. It is also clear that overexpression of specific IRS adaptor proteins within a cell line cannot “force” an activated IGFR1 to participate in a biological process that the cell does not normally display. For example, although IGFR1 is the predominant signaling pathway activated in a proliferative pathway in MCF-7 cells, overexpression of IRS-1 in cell lines that show no mitogenic response to IGF-I does not result in IGF-1-mediated proliferation (43).

Thus, it is likely that interaction of IGFR1 with other signaling pathways ultimately determines the biological consequences of IGF action in breast cancer cells. The clearest example of this hypothesis concerns the cross-talk between IGFR1 and estrogen receptor α in breast cancer cells (4). Recent work has shown that the IGFR1 signaling pathway depends on functional expression of estrogen receptor α (44). In this study, we show that inhibition of MDA-231BO cell migration by rhBP-1 could be because of dual effects, neutralization of IGF-I action, and interruption of integrin function. A better understanding of the molecular mechanisms by which IGFR1 and integrins interact in the metastatic cascade will be very important in designing new prevention and therapeutic approaches to breast cancer.

REFERENCES


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