Insulin-like Growth Factor-1 Induces Adhesion and Migration in Human Multiple Myeloma Cells via Activation of β1-Integrin and Phosphatidylinositol 3'-Kinase/AKT Signaling

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ABSTRACT

Insulin-like growth factor-1 (IGF-I) is a growth and survival factor in human multiple myeloma (MM) cells. Here we examine the effect of IGF-I on MM cell adhesion and migration, and define the role of β1 integrin in these processes. IGF-I increases adhesion of MM.1S and OPM6 MM cells to fibronectin (FN) in a time- and dose-dependent manner, as a consequence of IGF-IR activation. Conversely, blocking β1 integrin monoclonal antibody, RGD peptide, and cytochalasin D inhibit IGF-I-induced cell adhesion to FN. IGF-I rapidly and transiently induces association of IGF-IR and β1 integrin, with phosphorylation of IGF-IR, IRS-1, and p85βγ. IGF-I also triggers phosphorylation of Akt and ERK significantly. Both IGF-IR and β1 integrin colocalize to lipid rafts on the plasma membrane after IGF-I stimulation. In addition, IGF-I triggers polymerization of F-actin, induces phosphorylation of p125γAK and paxillin, and enhances β1 integrin interaction with these focal adhesion proteins. Importantly, using pharmacological inhibitors of phosphatidylinositol 3'-kinase (PI3-K) (LY294002 and wortmannin) and extracellular signal-regulated kinase (ERK) inhibitors, as well as cytochalasin D abrogate IGF-I-induced MM cell transmigration. Finally, IGF-I induces adhesion of CD138+ patient MM cells. Therefore, these studies suggest a role for IGF-I in trafficking and localization of MM cells in the bone marrow microenvironment. Moreover, they define the functional association of IGF-IR and β1 integrin in mediating MM cell homing, providing the preclinical rationale for novel treatment strategies targeting IGF-I/IGF-IR in MM.

INTRODUCTION

MM is a B-cell malignancy characterized by excess expansion of malignant plasma cells in the BM in close association with stromal cells. Their localization in the BM results from migration or “homing” of MM cells from the vascular to the extravascular compartment of the BM. Little is known about the mechanisms of this homing; nevertheless, their trafficking to the BM, as well as inside the BM microenvironment, is mediated by cell surface adhesion molecules at multiple steps. A complex network of cytokines and cell adhesion molecules is produced by BM stromal cells that regulate the proliferation, survival, and functional program of MM cells. For example, FN, a major ECM, is abundantly found within the BM microenvironment, and adhesion to FN in vitro can be inhibited by mAb to the integrins and by synthetic peptides that mimic the FN-binding sites (e.g., RGD; Ref. 1).

Integrins are α/β-heterodimeric membrane proteins that mediate cell adhesion to the ECM. Integrin ligand binding by ECMs induces cytoskeletal rearrangement and cell motility in a variety of cell types. In the absence of β1 integrins, hematopoietic stem cells have impaired migration (2). Although integrin-mediated adhesion is necessary for tumor motility, it is not sufficient. In human MM cells, the integrin α4β1 is one of the main adhesion receptors that mediate tumor cell binding to FN and vascular cell adhesion molecule (3, 4). β1-Integrin-mediated adhesion of MM cells to FN confers protection against drug-induced apoptosis (5, 6) and triggers nuclear factor κB-dependent transcription, and secretion of the major MM growth and survival factor IL-6 (7). We demonstrated recently costimulation of human MM cells via vascular endothelial growth factor and β1 integrin (8), supporting β1 integrin inside-out signaling. The IGF-I is a low molecular weight, single chain polypeptide of which the extensive local production is consistent with its mediating autocrine or paracrine growth, in addition to its more classical endocrine mechanism. IGF-I elicits its action on cells by binding to the IGF-IR, which consists of an α- and β-subunit heterodimer: ligand-dependent tyrosine kinase activity rests in the β-subunit. We and others have shown that IGF-I is a potent growth and survival factor in human MM cells (9–14). Specifically, IGF-I activates at least 2 distinct PI3-K and MAPK signaling pathways, leading to both proliferative and antiapoptotic effects (11–14). The increased growth of these cells in the presence of IGF-I requires IGF-IR and can be blocked by a neutralizing αIR3 mAb. IGF-I is also a BM stroma-derived chemoattractant factor for the murine 5T2 MM cells (15). To date, however, whether IGF-I plays a role in regulating human MM cell adhesion and migration within the BM microenvironment remains undetermined.

Cholesterol-rich microdomains of the plasma membrane, also termed lipid rafts, are implicated in the recruitment of essential proteins for intracellular signal transduction. Therefore, they provide signaling platforms to coordinate cellular adhesion (16, 17) and transmembrane signaling (18, 19). In human adenocarcinoma MCF-7 cells, transient redistribution of IGF-IR from nonraft to raft was observed after IGF-I treatment (18, 20). Membrane compartmentalization between rafts and nonrafs is also required for T-cell activation (21), and T-lymphocyte costimulation is mediated by reorganization of membrane raft microdomains (22). Importantly, integrins are lipid raft

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3 The abbreviations used are: MM, multiple myeloma; BM, bone marrow; IGF, insulin-like growth factor; IGF-IR, type-I IGF receptor; FN, fibronectin; ECM, extracellular matrix; IRS-1, insulin receptor substrate-1; PI3-K, phosphatidylinositol 3'-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated mitogen-activated protein kinase; mAb, monoclonal antibody; cyt D, cytochalasin D; PMA, phorbol myristate acetate; PLL, poly-l-Lysin; wort, wortmannin; Ab, antibody; chx, cycloheximide; F-actin, filamentous actin.
associated (16, 17, 23), although the functional relevance of this association remains undefined.

PI3-K and p125 FAK have been implicated in integrin-mediated cell motility in breast cancer cells and smooth muscle cells triggered by IGF-I (24, 25). Recent reports have demonstrated tyrosine phosphorylation of paxillin and p130CAS, and association of Crk with p130CAS after IGF-I stimulation in Swiss 3T3 cells (26), suggesting that IGF-I cross-regulates integrin-dependent signaling pathways. In addition, tumor cell metastasis is regulated by the functional cooperation between IGF-I signaling and integrin αvβ3, independent of tumor cell growth (27). These findings suggest that integrin ligation, in conjunction with cytokine activation, may play an important role in the dissemination of malignant tumor cells (27, 28). Because there is a lack of studies of IGF-I/IGF-IR and its interaction with cytokine activation, may play an important role in the determination of Distribution of Proteins between the Detergent-soluble and -insoluble Fractions. Serum-starved OPM6 cells (4 × 10^6/ml) were stimulated with IGF-I (100 ng/ml) for 0, 5, and 30 min before lysis in 200 μl of ice-cold buffer [1% Brij 58, 20 mM Tris (pH 7.5), and 150 mM NaCl with protease and phosphatase inhibitors; Ref. 30]. Brij 58, like Triton X-100, is a relatively mild, nonionic detergent. Membrane lipid rafts are insoluble in nonionic detergents at low temperature. The lysates were centrifuged at 14,000 × g for 25 min at 4°C, and the resultant supernatant was the detergent-soluble fraction (S). The detergent-insoluble pellets were resuspended and sonicated briefly in lysis buffer supplemented with 0.5% SDS and 1% β-mercaptoethanol. After centrifugation, the resulting supernatant was the detergent-insoluble fraction (I) of the cell, excluding the insoluble cytoskeletal fraction. This method separates proteins bound to membrane rafts (detergent-insoluble) from those that are raft-associated (detergent-soluble; Refs. 18, 30). The proteins were either separated directly by SDS-PAGE and then visualized by immunoblotting for IGF-IR, p85PI3-K, and Src, or first immunoprecipitated with an anti-β1 integrin mAb and then immunoblotted.

**Copatching Experiments.** Membrane lipid raft aggregation or patching was performed as described previously (18, 22). Serum-starved OPM6 cells, either treated with or without 100 ng/ml IGF-I for 10 min, were fixed in 2% paraformaldehyde for 20 min on ice, incubated with FITC-labeled cholera toxin B (FITC-CTx; 8 μg/ml) for 20 min, and then stained with either anti-β1 integrin (10 μg/ml) or anti-IGF-IR at 4°C for 30 min. Primary Abs were detected by Alexa Fluor 647 or Alexa Fluor 488-conjugated secondary Ab for 30 min on ice. After three washes, cells were layered on glass coverslips, fixed in 4% formaldehyde in PBS, and mounted onto slides in antifade solution (Molecular Probes). Cells were visualized using a Zeiss model LSM410 confocal laser scanning microscope (Zeiss, New York, NY) equipped with an external argon-krypton laser (488 and 568 nm) to detect colocalization of IGF-IR (red) and GM1 (green), as well as β1 integrin (red) and GM1 (green). Images of optical sections (512 × 512 pixels) were digitally recorded. The resulting images were processed using Adobe Photoshop software (Mountain View, CA).

**Actin Polymerization.** Serum-starved cells were treated with or without 100 ng/ml IGF-I, permeabilized, fixed, and stained in a single step by the addition of a 1-ml solution containing 0.1 mg/ml 1-lysophosphatidylcholine, 5% formaldehyde in PBS/1% BSA, and 5 units of Alexa Fluor 647-conjugated phalloidin (Molecular Probes). Cells were incubated for 20 min at 4°C, washed, and subjected to flow cytometry. F-actin content induced by IGF-I was expressed as a percentage of control by dividing the mean fluorescence intensity of cells at each time point to mean fluorescence intensity of the unstained cells at time 0.

**Transmigration Assay.** Chemicon QCM 96-well migration assay (8-μm pore size with FN-coated filter; Chemicon) was performed using cells pre-treated with indicated inhibitors for 60 min at 37°C. Cells (2 × 10^4/100 μl) were placed in the migration chamber, and 150 μl of adhesion medium with or without IGF-I (100 ng/ml) were added to the feeder tray. After 4-h incubation, 150 μl of lysis buffer/dye solution was added to each well of the feeder tray and incubated for 15 min. Plates were measured with a fluorescence plate reader using 480/520 nm filter set (Perkin-Elmer Life Sciences). Some serum-starved MM cell lines were preincubated for 1 h with or without mAbs against IGF-IR, β1 integrin, and MOPC21 control IgG1, cyt D, as well as chx. Transmigration assays were also performed as described previously (29).

**Statistics.** Data are mean ± SE. Statistical analysis used the Student’s t test, with P < 0.05 considered significant.

**RESULTS**

Effect of IGF-I on MM Cell Adhesion to FN. We first investigated whether IGF-I has an effect on MM cell adhesion to FN. In serum-starved MM cell lines MM.1S and OPM6, IGF-I (100 ng/ml) triggered increased MM cell adhesion as early as 2 min, which
reached maximum at 30 min (Fig. 1A). IGF-I at 20–400 ng/ml, physiological achievable concentrations, was next used in dose-response adhesion experiments. IGF-I significantly enhanced cell adhesion in a dose-dependent manner, with maximal adhesion at 100 and 40 ng/ml of IGF-I for MM.1S (1.7–2.3 fold increase) and OPM6 (2.5–3 fold increase) MM cells, respectively (Fig. 1B). As shown in Fig. 1C, inhibition of IGF-IR by αIR3 mAb significantly decreased adhesion compared with control mAb: 55–63% versus 0% inhibition for αIR3 mAb versus isotype control MOPC21 IgG. This confirms that IGF-I induces increased MM cell adhesion to FN via IGF-IR.

Anti-β1 Integrin mAb and RGD Peptide Block IGF-I-enhanced MM Adhesion to FN. Because β1 integrin mediates MM cell adhesion to FN and BM stromal cells, we next investigated the role of β1 integrin in IGF-I-enhanced MM cell adhesion. To determine whether β1 integrin mediates IGF-I-induced cell-ECM adhesion, blocking anti-β1 integrin mAb or RGD peptide (100 μM), respectively, were added together with 100 ng/ml IGF-I. As shown in Fig. 2, anti-β1 integrin mAb (5–20 μg/ml) and RGD peptide (100 μM) significantly decreased IGF-I-stimulated MM cell adhesion, whereas isotype control IgG and the mutant RGE peptide had no effect. In addition, pretreatment of MM cells with cyt D (0.5 μM), an inhibitor of actin filament polymerization, completely blocked IGF-I-induced adhesion, indicating that cytoskeletal proteins are involved in β1 integrin-mediated MM cell adhesion induced by IGF-I.

IGF-I Induces Transient Association of IGF-IR and β1 Integrin. We next examined IGF-I/IGF-IR signaling in MM.1S and OPM6 cells. Specifically, serum-starved cells were treated with IGF-I for up to 1 h and analyzed for tyrosine phosphorylation, and activation of IGF-IR and its substrate IRS-1, as well as activation of the PI3-K/AKT and MAPK pathways (Fig. 3A). Tyrosine phosphorylation of IGF-IR was maximal within 2 min and decreased after 20 and 10 min in MM.1S and OPM6 cells, respectively, confirming receptor autophosphorylation and internalization induced by IGF-I. Tyrosine phosphorylation of IRS-1, association of IRS-1 with p85PI3-K, and phosphorylation of AKT were detectable within 2 min and persisted for at least 60 min (Fig. 3A). Phosphorylation of ERK1/2 was increased at 2 min and remained above basal levels after 60 min of IGF-I treat-
attachment of cells to FN (31, 32), we next prepared cell extracts from MM.1S or OPM6 cells were treated with IGF-I for the indicated times. Cell lysate (500 μg) was immunoprecipitated with anti-IGF-IR and anti-IRS-1 Abs and then immunoblotted with anti-pTyr mAb. Membranes were stripped and reprobed with anti-IGF-IR, anti-β1 integrin, and anti-pS(416) PI3-K Abs. A total of 60 μg of cell lysate was resolved by 8% SDS-PAGE, and immunoblotted with anti-pAKT and anti-pERK Abs. α-tubulin serves as loading controls. B, serum-starved cells were incubated with IGF-I (100 ng/ml) or PMA (100 ns) for 10 min or pretreated with αRI3 (5 μg/ml) for 30 min before incubation with IGF-I (100 ng/ml) for 10 min. One ng of cell lysates was immunoprecipitated with 4 μg of anti-β1 integrin Abs. Immunoprecipitates were analyzed by immunoblotting with anti-IGF-IR and anti-β1 integrin Abs.

Fig. 3. IGF-I stimulates association of IGF-IR and β1 integrin, as well as activation of PI3-K/AKT and ERK pathways. A, serum-starved MM.1S or OPM6 cells were treated with IGF-I for the indicated times. Cell lysate (500 μg) was immunoprecipitated with anti-IGF-IR and anti-IRS-1 Abs and then immunoblotted with anti-pTyr mAb. Membranes were stripped and reprobed with anti-IGF-IR, anti-β1 integrin, and anti-pS(416) PI3-K Abs. A total of 60 μg of each lysate was also resolved by 8% SDS-PAGE, and immunoblotted with anti-pAKT and anti-pERK Abs. α-tubulin serves as loading controls. B, serum-starved cells were incubated with IGF-I (100 ng/ml) or PMA (100 ns) for 10 min or pretreated with αRI3 (5 μg/ml) for 30 min before incubation with IGF-I (100 ng/ml) for 10 min. One ng of cell lysates was immunoprecipitated with 4 μg of anti-β1 integrin Abs. Immunoprecipitates were analyzed by immunoblotting with anti-IGF-IR and anti-β1 integrin Abs.

ment. Importantly, anti-IGF-IR immunoprecipitates from cell extracts immunoblotted with anti-β1 integrin mAbs demonstrated IGF-IR and β1 integrin complex formation within 2 min. This interaction of β1 integrin and IGF-IR was transient, occurring within 2 min and decreasing after 20 min of IGF-I treatment (Fig. 3A). Because PMA is a strong agonist for the adhesiveness of β1 integrins and regulates attachment of cells to FN (31, 32), we next prepared cell extracts from MM cells after 10 min treatment with PMA (100 ns) and IGF-I (100 ng/ml), and then immunoprecipitated with anti-β1 integrin mAb. As shown in Fig. 3B, IGF-I was detected in anti-β1 integrin immunoprecipitates after either treatment. In addition, pretreatment with αRI3 mAb inhibits coimmunoprecipitation of β1 integrin with IGF-IR (Fig. 3B). These data confirm that IGF-IR associates with β1 integrin in MM cells and additionally suggest that these receptors may cross-regulate one another in response to IGF-I or PMA.

IGF-IR and β1 Integrin Are Colocalized in Membrane Lipid Rafts upon IGF-I Treatment. To evaluate the functional significance of the association of IGF-IR with β1 integrin, we next examined whether they are colocalized in membrane rafts after IGF-I treatment in OPM6 MM cells. Membrane rafts, microdomains of the plasma membrane enriched in cholesterol and sphingolipids, are insoluble after treatment with nonionic detergents such as Triton X-100 and Brij58 at low temperatures (18, 23, 30). We examined the distribution of complex components between the detergent-soluble (S) and detergent-insoluble (I) fractions of OPM6 MM cells, before and after IGF-I stimulation and protein extraction at 4°C. As shown in Fig. 4A, IGF-IR and β1 integrin are present mainly in the S fraction in serum-starved OPM6 before IGF-I treatment, but are primarily in the I fraction after IGF-I stimulation. p85S(416)-K, one of the earliest substrates activated by the IGF-IR, is similarly induced in the I fraction. Thirty min after IGF-I stimulation, these proteins return from membrane rafts (I fraction) to nonraffs (S fraction), demonstrating the temporal sequence and dynamics of their association with membrane rafts and their functional role in mediating IGF-I-stimulated MM cell adhesion and subsequent migration. To confirm that proteins in I fraction had been separated from proteins in S fraction, we immunoblotted for Src using a pan-Src Ab. Because Src is associated exclusively with membrane rafts (33), constitutive expression of Src in the I fraction but not in the S fraction confirms the quality of the preparation (Fig. 4A). These data demonstrate that membrane rafts are essential for IGF-I/IGF-IR signaling (18, 19), and additionally show IGF-I-induced migration of β1 integrin from nonraffs to rafts on the cell membrane.

We next validated IGF-I-triggered recruitment of these proteins to rafts in intact cells using confocal microscopy. Aggregated lipid rafts can be detected as distinct patches by clustering of raft markers with Abs or other reagents (21). Upon coalescence of lipid rafts into larger domains, other raft-associated proteins will colocalize with these patches. Nonraft-associated proteins do not colocalize with raft patches, because of the solubility of the different lipid phases. Therefore, serum-starved and IGF-I-treated OPM6 cells were costained with anti-β1 integrin mAb (red) and with CTx (green). CTx, which binds specifically to raft-ganglioside GM1, was used to visualize membrane rafts. Cells before and after IGF-I treatment were also costained with anti-IGF-IR mAb (red) and with CTx (green). These experiments using anti-β1 integrin mAb and CTx showed little, if any, overlap between β1 integrin and GM1 in unstimulated OPM6 cells (Fig. 4B). However, stimulation with IGF-I increased β1 integrin costained with CTx (yellow), indicating that IGF-I stimulates β1 integrin and raft clustering in the cell membrane (Fig. 4B). In parallel, IGF-IR colocalization with CTx after IGF-I stimulation is even more distinct in IGF-I-treated versus control unstimulated cells (Fig. 4B). These results demonstrate the association of IGF-IR and β1 integrin with membrane rafts after IGF-I stimulation in intact OPM6 MM cells. Furthermore, when lipid rafts were disrupted using methyl-β-cyclodextrin (5 mM) to deplete cholesterol, IGF-I-induced adhesion was completely blocked to the baseline (data not shown). Conversely, replenishment with cholesterol (30 μg/ml) after methyl-β-cyclodextrin treatment completely restores IGF-I-enhanced adhesion. Therefore, β1 integrin-mediated adhesion induced by IGF-I depends on intact membrane rafts.

IGF-I Induces β1 Integrin Interaction with Activated Signaling Proteins Localized at Sites of Focal Adhesion. Because pretreatment with cyt D abrogates IGF-I-enhanced MM adhesion to FN (Fig. 2), we next asked whether IGF-I increased levels of F-acitin in OPM6 cells. We observed that IGF-I triggers a rapid increase in the polymerization of F-acitin; it is detected as early at 2 min, persists for at least 10 min, and decreases afterward (Fig. 5). The increased actin polymerization occurs during IGF-I-enhanced adhesion.

We next examined whether focal adhesion formation because of β1
integrin-dependent intracellular signaling proteins (i.e. p125FAK and paxillin), associated with actin polymerization, is influenced by IGF-I. Tyrosine phosphorylation of these proteins was evaluated by immunoprecipitation of OPM6 cell lysates using indicated Abs, followed by anti-pTyr immunoblotting. Fig. 6A shows that maximum phosphorylation of p125 FAK and paxillin is evident 10–20 min after IGF-I treatment. There is a time lag of at least 10 min between maximal IGF-IR and p125 FAK/paxillin phosphorylation. The temporal dependence of phosphorylation may represent sequential events during IGF-I-controlled MM adhesion and migration. Conversely, IGF-IR blocking mAb H9251IR3 inhibits the IGF-I-induced tyrosine phosphorylation of both p125 FAK and paxillin (Fig. 6B). In the whole cell extracts, the IGF-IR β subunit is clearly visible as Mr 97,000 band, and aIR3 mAb blocks IGF-I stimulation of IGF-IR tyrosine phosphorylation and subsequent activation of p125 FAK and paxillin (Fig. 6B).

To additionally examine whether IGF-I enhances physical interaction of β1-integrin with p125 FAK and paxillin, serum-starved OPM6 cells were cultured on FN-coated plates, and then treated with or without IGF-I for 10 min. Cell extracts were prepared for immunoprecipitation with anti-β1 integrin mAb followed by immunoblotting with anti-p125 FAK, anti-α-actinin, and anti-paxillin Abs. Fig. 6C shows that β1 integrin interacts with p125 FAK, α-actinin, and paxillin in OPM6 cells adherent to FN. The interaction between β1 integrin and these focal adhesion proteins is not observed in cells adherent to PLL, which...
increases cell adhesion to plastics through nonspecific binding (data not shown). Importantly, the interaction of β1 integrin with focal adhesion components is enhanced in OPM6 cells after treatment with IGF-I; by densitometric analysis, β1 integrin coimmunoprecipitation with p125 FAK (59%), α-actinin (47%), and paxillin (62%) increases, compared with adhesion of untreated MM to FN (Fig. 6D). Fig. 6E additionally shows that pretreatment with 0.5 μM cyt D for 30 min completely abrogates p125 FAK/paxillin tyrosine phosphorylation induced by IGF-I and the baseline activation of these proteins in OPM6 cells adherent to FN. These data indicate that IGF-I cross-regulates activation of β1-integrin signaling, including phosphorylation of p125 FAK and paxillin.

**Effects of Inhibitors of PI3-K and ERK on IGF-I-induced β1 Integrin-dependent MM Adhesion and Transmigration.** Because IGF-I predominantly induces PI3-K/AKT and ERK1/2 pathways in MM.1S and OPM6 cells (Fig. 3A), we next determined whether these pathways mediate IGF-I-enhanced adhesion and transmigration of MM cells using PI3-K and ERK1/2 inhibitors. As shown in

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**Fig. 6.** IGF-I enhances β1 integrin interaction with activated focal adhesion proteins. A, serum-starved OPM6 cells were treated for 0–40 min with 100 ng/ml of IGF-I. Immunoprecipitation was performed using indicated Abs followed by immunoblotting with anti-pTyr mAb. Direct immunoblotting with anti-p125 FAK and anti-paxillin Abs serves as loading controls. B, OPM6 cells were pretreated for 30 min, with or without 5 μg/ml αIR3. Tyrosine phosphorylation was assessed 10 min after IGF-I (100 ng/ml) treatment. IGF-IR tyrosine phosphorylation was assessed in whole-cell lysates because the addition of αIR3 mAb precluded IGF-IR immunoprecipitation. C, serum-starved OPM6 cells were plated on dishes coated with FN, and treated with or without IGF-I (100 ng/ml) for 10 min. Attached cells were lysed and immunoprecipitated with anti-β1 integrin (β1) mAb and with normal IgG serum control. Immunoprecipitates were analyzed by immunoblotting with anti-p125 FAK, anti-α-actinin, and anti-paxillin Abs. D, lysates from OPM6 cells on FN-coated plates, pretreated with or without cyt D (0.5 μg/ml), and then stimulated with or without 100 ng/ml IGF-I for 10 min, were immunoprecipitated with anti-p125 FAK and paxillin Abs, followed by immunoblotting with anti-pTyr mAb. Results are representative of two independent experiments.

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**Fig. 7.** IGF-I enhances β1 integrin-mediated MM adhesion in a PI3-K-dependent manner. A, MM.1S (●) and OPM6 (■) cells were pretreated with or without PI3-K inhibitors (LY294002 or wort) or ERK inhibitor (PD98059), and then incubated with or without IGF-I (100 ng/ml, +) for 15 min. Adhesion data are the mean of triplicate samples from one representative of three independent experiments; bars, ±SE. *, statistical significance (P < 0.05) of inhibition of adhesion in IGF-I-treated cells pretreated without inhibitors versus controls. B, before cell attachment to uncoated-, PLL-coated, or FN-coated plates, MM.1S and OPM6 cells were preincubated with or without LY294002. Adhesion was assessed in the presence or absence of IGF-I (100 ng/ml). Data are representative of three independent experiments; *, statistical significance (P < 0.05) of inhibition of cells added to FN versus PLL.
Fig. 7A, pretreatment with PI3-K inhibitors LY294002 (25–50 μM) or wort (0.1–0.3 μM) for 1 h completely prevents IGF-I-induced MM adhesion to FN, whereas pretreatment with ERK1/2 inhibitor PD98059 has no effect. In the presence of LY294002 (25–50 μM), PD98059 does not additionally inhibit IGF-I-induced MM cell adhesion. Thus, IGF-I-induced MM adhesion depends on activation of PI3-K/AKT signaling. We also examined the effect of LY294002 on IGF-I-induced MM adhesion to uncoated, PLL-coated, or FN-coated plates. Preincubation of MM.1S and OPM6 cells with LY294002 (20–50 μM) completely abolishes the increased adhesion to FN induced by IGF-I (Fig. 7B). Importantly, treatment of MM cells with LY294002 (25 or 50 μM) does not affect cell viability or cell adhesion on uncoated or PLL-coated plates (Fig. 7B). These results demonstrate that IGF-I regulates β1 integrin-mediated MM adhesion in a PI3-K-dependent manner.

We next measured the effect of LY294002 or wort and PD98059 on IGF-I-induced MM cell transmigration. Pretreatment with LY294002 or wort for 1 h significantly inhibits the increase in MM cell transmigration induced by IGF-I (Fig. 8A). In contrast, pretreatment with PD98059 has little effect on IGF-I-induced MM transmigration, confirming that PI3-K/AKT is a key regulator in this process. In addition, pretreatment with αR3 mAb, cyt D, and anti-β1 integrin mAb abrogates IGF-I-induced MM transmigration (Fig. 8B). Interestingly, IGF-I-induced MM transmigration does not require de novo protein synthesis, because chx pretreatment (chx; 5 μg/ml) for 30 min does not significantly abrogate the increased MM transmigration induced by IGF-I.

IGF-I Induces Adhesion of CD138+ MM Cells to FN. Finally, we determined whether IGF-I induces adhesion of CD138+ patient MM cells. CD138+ MM cells from three patient samples were incubated with or without IGF-I (200 ng/ml) and then added to FN-coated plates. IGF-I induces a 1.4–2.8-fold increase in adherence to FN; conversely, pretreatment of αR3 diminishes IGF-I-induced adhesion (Fig. 9). Because we did not serum-starve freshly isolated CD138+ patient MM cells, αR3 mAb does not completely abolish IGF-I-induced adhesion to FN. It is likely that other factors in the serum, i.e., vascular endothelial growth factor, contribute to MM cell adhesion to FN.

**DISCUSSION**

We and others have defined the central role of PI3-K/AKT signaling in mediating IGF-I-induced MM cell proliferation and apoptosis (11, 13, 14). In the present study, we show that activation of PI3-K/AKT and β1 integrin signaling cascades are key regulators in IGF-I-induced cell adhesion, and transmigration in MM.1S and OPM6 MM cells. We demonstrate that IGF-I stimulates adhesion and migration of human MM cells via IGF-IR. We show that IGF-I induces rapid and transient association of activated IGF-IR and β1 integrin, and additionally define that association of IGF-IR and β1 integrin in membrane rafts is critical for IGF-I-induced cell adhesion. IGF-I activates PI3-K/AKT and ERK signaling, as well as actin polymerization and focal adhesion complex formation including p125FAK and paxillin. IGF-I additionally enhances the interaction of β1 integrin with these activated focal adhesion signaling molecules; conversely, inhibition of actin polymerization with cyt D abrogates...
such interactions, confirming the importance of concomitant activation of IGF-I and β1 integrin signaling in mediating IGF-I-induced cell adhesion and migration. Because IGF-I-induced adhesion and migration is completely blocked in the presence of specific PI3-K inhibitors, but not ERK inhibitors, we conclude that IGF-I-stimulated adhesion and migration is dependent on PI3-K/AKT activity. Finally, IGF-I induces adhesion of CD138+/ patient MM cells, whereas dI3 mAb blocks this response. Taken together, our results suggest that IGF-IR and β1 integrin play coordinated roles in enhancing MM cell adhesion in the BM milieu, supporting the preclinical rationale for targeting IGF-I/IGF-IR in novel treatment strategies for MM.

Our data extend a previous report of IGF-I as a chemoattractant in the mouse 5T2 MM model (15, 34, 35) to human MM cells, providing new insights into the role of IGF-I/IGF-IR in MM pathogenesis and clinical progression. This pleiotropic growth factor is produced in BM stromal cells, MM cells, osteoblasts, and endothelial cells in the BM milieu. Our results support a role for IGF-I in MM cell progression, as proposed in a recent clinical study showing that MM patients with higher serum IGF-I levels have inferior survival (36).

Because IGF-I induces MM cell adhesion very rapidly, early IGF-IR signaling events could be decisive. IGF-I stimulation induces transient association between IGF-IR and β1 integrin (Fig. 3), which is consistent with a previous report (37). This association could be either direct or via integrin-associated protein (38). Importantly, a blocking anti-β1 integrin mAb completely blocks IGF-I-induced MM adhesion to FN, confirming a role for β1 integrin in these processes. PI3-K/AKT activity is induced after IGF-I stimulation; and conversely, IGF-I-induced MM cell adhesion and migration is inhibited by LY294002 and wort. Moreover, expression of dominant-negative AKT using adenovirus vectors completely blocks IGF-I-induced MM adhesion and migration (data not shown). Therefore, PI3-K/AKT activity mediates IGF-I-induced MM cell adhesion and migration. In our previous study, LY294002 inhibits phosphorylation of AKT but not of ERK1/2 induced by sCD40L, suggesting that PI3-K and MAPK pathways are independent (13, 29). Our present studies also indicate the independence of these two pathways. Interestingly, IGF-I-induced migration is not dependent on de novo protein synthesis, because cyclohexamide treatment has no effect on this response. The differential effects of sCD40L and IGF-I on migration may be attributable, at least in part, to differential activation of nuclear factor κB and its target genes.

IGF-I induces an increase in actin polymerization, therefore enhancing interactions among integrin, cytoskeletal, and signaling components. It contributes, at least in part, to IGF-I-induced MM adhesion. Increasing evidence supports a role for IGF-IR in regulating focal adhesion molecules, and we show here that p125Fak is tyrosine phosphorylated and activated by IGF-IR. Paxillin, which associates with p125Fak and is tyrosine phosphorylated upon p125Fak activation, is also tyrosine phosphorylated after IGF-I stimulation of MM cells. Because phosphorylation of these focal adhesion molecules is triggered by β1 integrin binding to FN, these results show that both IGF-I and β1 integrin activate the focal adhesion pathway. These results confirm that activated IGF-IR rapidly associates with β1 integrin after IGF-I treatment (Fig. 3, A and B), demonstrating the functional sequelae of costimulation of both receptors. Because there is a time lag between phosphorylation of p125Fak and paxillin, phosphorylation of p125Fak may be the initial event during cell adhesion, with subsequent paxillin phosphorylation followed by dephosphorylation of both molecules leading to cell migration. Furthermore, we observed enhanced phosphorylation of focal adhesion proteins after IGF-I treatment of MM cells adherent to FN-coated plates, which was completely abolished by cytochalasin D (Fig. 7D). Taken together, our data define a role for IGF-IR in the regulation of focal adhesion proteins critical for MM cell adhesion and migration. In addition, these results are in concert with a recent study showing that IGF-I-induced tyrosine phosphorylation of focal adhesion proteins can be dissociated from the activation of the ERK pathway in Swiss 3T3 cells (26).

Segregation between raft and nonraft proteins in unstimulated cells localizes molecules during cell adhesion and migration. Our data indicate that IGF-I triggers clustering of membrane rafts, which coalesce into large domains. Moreover, recruitment of activated integrins into lipid rafts facilitates upstream cell signaling. Although β1 integrins may associate with lipid rafts (16, 17, 39), they may be restrained by cytoskeletal tethering in a manner that excludes them from lipid rafts under unstimulated conditions. In our study, β1 integrin is located predominantly in the soluble fraction of lysates from unstimulated cells and stains mainly in the nonrafts of unstimulated cell membranes. IGF-I induces rapid and transient untethering of β1 integrin from the cytoskeleton, its migration into membrane rafts, and its binding again to the cytoskeleton during MM cell adhesion and migration. Because IGF-I also stimulates actin polymerization, optimal interaction of β1 integrin and activated focal adhesion signaling proteins is achieved to ensure these processes. These results demonstrate a complex dynamic sequence of events during cell migration induced by IGF-I in human MM cells, and confirm that cell adhesion is a prerequisite for cell migration. In addition, disruption of rafts by chemical depletion of membrane cholesterol impairs IGF-I-induced cell adhesion, confirming a role of membrane rafts in IGF-I/IGF-IR and β1 integrin signaling. These functional sequelae of lipid rafts in MM cell adhesion are in concert with studies using T cells (16, 21, 22) and breast cancer cells (18, 20). Furthermore, Semac et al. (40) showed recently that Rituximab (mAb targeting CD20) in Burkitt lymphoma-derived Raji cells, by redistributing CD20 to rafts, modifies their stability and organization, thereby modulating associated signaling pathways and C’ defense capacity. Our study suggests that targeting IGF-I/IGF-IR using a humanized IGF-I/IR Ab or small molecule may have similar functional sequelae.

In conclusion, our study identifies a chemotactic effect of IGF-I on human MM cells mediated via activation of PI3-K/AKT and β1 integrin. We demonstrate for the first time that membrane raft association with IGF-IR and β1 integrin regulates MM cell adhesion and migration within the BM milieu. Importantly, these results suggest a role for IGF-I in MM disease progression and support targeting IGF-I/IGF-IR in novel MM treatment strategies.

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REFERENCES


Announcements

(Requests for announcements must be received at least three months before publication.)

FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

2004 March 27–31, Orlando, FL
2005 April 16–20, Anaheim, CA

AACR SPECIAL CONFERENCES IN CANCER RESEARCH

A number of meetings are now being organized in the AACR’s series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, 615 Chestnut Street, 17th Floor, Philadelphia, PA 19106-4404. Up-to-date program information is also available via the Internet at the AACR’s website (http://www.aacr.org).

AACR-NCI-EORTC INTERNATIONAL CONFERENCE ON MOLECULAR TARGETS AND CANCER THERAPEUTICS

November 17–21, 2003
Hynes Center, Boston, MA

Chairpersons
Charles L. Sawyers, Los Angeles, CA
Edward A. Sausville, Bethesda, MD
Jaap Verweij, Rotterdam, The Netherlands

SIXTH JOINT CONFERENCE OF THE AACR AND JCA, ADVANCES IN CANCER RESEARCH

January 25–29, 2004
Hilton Wai Koloa Village, Wai Koloa, Hawaii

Chairpersons
Waun Ki Hong, Houston, TX
Takahashi Tsuruo, Tokyo, Japan

CALENDAR OF EVENTS


10th Hong Kong International Cancer Congress, November 19–21, 2003, Faculty of Medicine Building, The University of Hong Kong, Hong Kong. Contact: 10th HKICC Congress Secretariat, Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232 or 852.2855.4235; Fax: 852.2818.1186; E-mail: mededcon@hku.hk; Website: www.hkicc.org.

Third International Conference and 9th Annual Meeting of the International Society of Cancer Chemoprevention (ISCaC): Controversies in Tumor Prevention and Genetics, February 12–14, 2004, University of St. Gallen, Switzerland. E-mail: info@oncoconferences.ch; Website: www.oncoconferences.ch.


Molecular Targets for Cancer Therapy: 3rd Biennial Meeting, October 1–5, 2004, Don Cesar Beach Resort & Spa, St. Petersburg Beach, FL. Contact: Ann Gordon. Phone: 813.903.4975; E-mail: gordonac@moffitt.usf.edu.
Corrections

In the article by T. Wissniowski et al., titled “Activation of Tumor-specific T Lymphocytes by Radio-Frequency Ablation of the VX2 Hepatoma in Rabbits,” which appeared in the October 1, 2003 issue of *Cancer Research* (pp. 6496–6500), the names of the first two authors were misspelled. The correct author list is as follows: Thaddäus Till Wissniowski, Johannes Hänsler, Daniel Neureiter, Markus Frieser, Stefan Schaber, Birgit Esslinger, Reinhard Voll, Deike Strobel, Eckhart Georg Hahn, and Detlef Schuppan.

In the article by B. Ruggeri et al., titled “CEP-7055: A Novel, Orally Active Pan Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases with Potent Antiangiogenic Activity and Antitumor Efficacy in Preclinical Models,” which appeared in the September 15, 2003 issue of *Cancer Research* (pp. 5978–5991), two co-authors, Shi Yang (Department of Biochemistry, Cephalon, Inc.) and Sonya Pritchard (Department of Oncology, Cephalon, Inc.), were inadvertently omitted from the list of authors. The correct list is as follows: Bruce Ruggeri, Jasbir Singh, Diane Gingrich, Thelma Angeles, Mark Alburn, Shi Yang, Hong Chang, Candy Robinson, Kathryn Hunter, Pawel Dobrzanski, Susan Jones-Bolin, Sonya Pritchard, Lisa Aimone, Andrea Klein-Szanto, Jean-Marc Herbert, Francoise Bono, Paul Schaeffer, Pierre Casellas, Bernard Bourie, Roberto Pili, John Issacs, Mark Ator, Robert Hudkins, Jeffrey Vaught, John Mallamo, and Craig Dionne.

In the article by Y-T Tai et al., titled “Insulin-like Growth Factor-1 Induces Adhesion and Migration in Human Multiple Myeloma Cells via Activation of β1-Integrin and Phosphatidylinositol 3’-Kinase/AKT Signaling,” which appeared in the September 15, 2003 issue of *Cancer Research* (pp. 5850–5858), the Western blotting in the bottom “IGF-1R” panel of figure 3B is incorrect. The correct figure appears below:

![Fig. 3. IGF-I stimulates association of IGF-IR and β1 integrin, as well as activation of PI3-K/AKT and ERK pathways. A, serum-starved MM.1S or OPM6 cells were treated with IGF-I for the indicated times. Cell lysate (500 μg) was immunoprecipitated with anti-IGF-IR and anti-IRS-1 Abs and then immunoblotted with anti-pTyr mAb. Membranes were stripped and reprobed with anti-IGF-IR, anti-β1 integrin, and anti-p85 PI3-K Abs. A total of 60 μg of each lysate was also resolved by 8% SDS-PAGE, and immunoblotted with anti-pAKT and anti-pERK Abs. α-tubulin serves as loading controls. B, serum-starved cells were incubated with IGF-I (100 ng/ml) or PMA (100 nM) for 10 min or pretreated with αIR3 (5 μg/ml) for 30 min before incubation with IGF-I (100 ng/ml) for 10 min. One mg of cell lysates was immunoprecipitated with 4 μg of anti-β1 integrin Abs. Immunoprecipitates were analyzed by immunoblotting with anti-IGF-IR and anti-β1 integrin Abs.

Fig. 3. IGF-I stimulates association of IGF-IR and β1 integrin, as well as activation of PI3-K/AKT and ERK pathways. A, serum-starved MM.1S or OPM6 cells were treated with IGF-I for the indicated times. Cell lysate (500 μg) was immunoprecipitated with anti-IGF-IR and anti-IRS-1 Abs and then immunoblotted with anti-pTyr mAb. Membranes were stripped and reprobed with anti-IGF-IR, anti-β1 integrin, and anti-p85 PI3-K Abs. A total of 60 μg of each lysate was also resolved by 8% SDS-PAGE, and immunoblotted with anti-pAKT and anti-pERK Abs. α-tubulin serves as loading controls. B, serum-starved cells were incubated with IGF-I (100 ng/ml) or PMA (100 nM) for 10 min or pretreated with αIR3 (5 μg/ml) for 30 min before incubation with IGF-I (100 ng/ml) for 10 min. One mg of cell lysates was immunoprecipitated with 4 μg of anti-β1 integrin Abs. Immunoprecipitates were analyzed by immunoblotting with anti-IGF-IR and anti-β1 integrin Abs.
In the article by G. Yousef et al., titled “Human Kallikrein 5: A Potential Novel Serum Biomarker for Breast and Ovarian Cancer,” which appeared in the July 15, 2003 issue of Cancer Research (pp. 3958–3965), figure 4 was printed incorrectly. Below is the correct figure.

**Fig. 4.** Fractionation of three biological fluids (serum, ascites fluid from an ovarian cancer patient, and breast milk) by size-exclusion liquid chromatography. The elution profile of molecular mass standards is denoted by arrows. In serum, hK5 elutes as two immunoreactive peaks, one with an apparent molecular mass of 50 kDa (fractions 37–39) and one with an apparent molecular mass of approximately 150–180 kDa (fractions 31–33). The elution profile of another kallikrein with a similar theoretical molecular mass, hK6, is also shown by dashed lines. This kallikrein elutes at a molecular mass of ~35 kDa, corresponding to free hK6. In ascites fluid, the same comments apply as for serum. In breast milk, hK5 elutes mainly as a single immunoreactive peak. hK6 elutes as two distinct peaks, one at a molecular mass of ~35 kDa and another one at a molecular mass of ~100 kDa.
Insulin-like Growth Factor-1 Induces Adhesion and Migration in Human Multiple Myeloma Cells via Activation of β1-Integrin and Phosphatidylinositol 3’-Kinase/AKT Signaling

Yu-Tzu Tai, Klaus Podar, Laurence Catley, et al.


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