

Insulin-like growth factor-I promotes migration in human androgen-independent prostate cancer cells via the $\alpha\text{v}\beta\text{3}$ integrin and PI3-K/Akt signaling

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Abstract. In its phase of androgen-independence, prostate carcinoma is characterized by a high proliferation rate and by a strong ability to give rise to metastases. IGF-I has been shown to exert a potent mitogenic action on prostate cancer. We investigated whether IGF-I might also affect the motility of prostate cancer cells and defined the mechanism of action. We found that IGF-I promotes the migratory capacity of androgen-independent prostate cancer cells through the activation of its specific receptor, IGF-IR. This effect was accompanied by a change in cell morphology (as revealed by scanning electron microscopy), and by a rearrangement of the actin cytoskeleton. The treatment of cells with the PI3-K inhibitor, LY294002, counteracted the pro-migratory activity of IGF-I. Experiments were then performed to clarify whether the integrin, $\alpha\text{v}\beta\text{3}$, could be involved in the action of IGF-I. We demonstrated that: a) the IGF-I-induced migration of cells is completely antagonized by an antibody specifically blocking the function of $\alpha\text{v}\beta\text{3}$; b) IGF-I increases $\alpha\text{v}\beta\text{3}$ immunofluorescence at the level of cell membranes, and this effect is counteracted by LY294002; and c) IGF-I increases $\alpha\text{v}\beta\text{3}$ protein levels. Our results demonstrate that IGF-I promotes the motility of androgen-independent prostate cancer cells by modulating $\alpha\text{v}\beta\text{3}$ integrin activation/expression; these effects are mediated by the PI3-K/Akt signaling pathway. This study: a) supports a crucial role for IGF-I in the progression of the pathology towards the highly metastatic phase; and b) provides an additional rationale basis for the development of therapeutic strategies directed at the IGF-I/IGF-IR system in the treatment of androgen-independent prostate cancer.

Introduction

Despite the intensive research aimed at identifying new and more successful therapeutical approaches, prostate cancer still remains the second cause of cancer deaths in males in Western countries (1). In its initial stages, prostate carcinoma is usually androgen-dependent, and the majority of patients positively respond to a therapy based on androgen ablation or blockade of androgen action (2). However, after a period of remission, almost invariably, the pathology progresses to a condition of androgen-independence (3) characterized by a high proliferation rate and by a strong ability to give rise to metastases (4,5). The therapeutical options for hormone-resistant prostate cancer are still very limited (6,7).

The insulin-like growth factor-I (IGF-I) system has been implicated in the control of growth of several common neoplasias (8). In particular, there is now strong evidence, both from experimental and from epidemiological studies, that a close association exists between IGF-I and IGF-I receptors (IGF-IR) and prostate cancer growth (9). Prostate cancer cells produce IGF-I, express IGF-IR and exhibit a significant responsiveness to the mitogenic activity of IGF-I *in vitro* (10-13), while IGF-IR inhibition by means of antisense RNA reduces prostate cancer cell proliferation (14). The androgen-dependent prostate cancer LNCaP cell line expresses the IGF-I system and responds to IGF-I with increased proliferation; when these cells are induced to progress to an androgen-independent condition, they exhibit increased expression and signaling of IGF-IR (15). In *in vivo* studies (performed in SCID mice), the progression of androgen-dependent prostate cancer xenografts (derived from the LAPC-9 and LNCaP cell lines) towards a phase of androgen-independence is associated with increased levels of both IGF-I and IGF-IR (16); similarly, prostate-specific IGF-I mRNA expression was found to increase during prostate cancer progression in TRAMP mice and to be elevated in the accompanying metastases (17). Finally, elevated serum levels of IGF-I have been reported to be associated with an increased risk of prostate cancer in men (18,19), and the level of expression of IGF-IR, which is up-regulated in primary prostate cancer, seems to persist in metastatic disease (20).

Taken together, these observations point to IGF-I as a crucial mitogenic factor involved in prostate cancer develop-

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ment and progression. However, although the proliferative and antiapoptotic effects of IGF-I in prostate cancer are now well established, the data on a possible direct effect of this growth factor on the metastatic behavior of prostate cancer cells are still scanty.

Metastasis is a complex process in which cancer cells detach from the primary tissue and acquire the ability to migrate towards target tissues where they give rise to colonies. Integrins, the heterodimeric proteins that mediate cell adhesion to the extracellular matrix, are known to be deeply involved in the processes of cell migration and invasion (21). In prostate cancer, the integrin dimer, $\alpha\beta3$, seems to be particularly involved in the progression of the pathology, being highly expressed in metastatic androgen-independent (PC3) but absent in less aggressive hormone-dependent (LNCaP) prostate cancer cells (22).

In the present study, we investigated the effects of IGF-I on the migratory behavior of androgen-independent prostate cancer cells; the involvement of the $\alpha\beta3$ integrin as well as the intracellular signaling pathway mediating the action of IGF-I have also been defined.

Materials and methods

Materials. Human recombinant IGF-I was purchased from Peptotech EC, Ltd. (London, UK) and neutralizing anti-IGF-IR mAb $\alpha\text{IR}3$ was obtained from Oncogene Research Products (San Diego, CA). Mouse anti- $\alpha\beta3$ antibody was from Chemicon (Temecula, CA). The phosphatidylinositol-3-kinase (PI3-K) inhibitor, LY294002; the MAP kinase inhibitor, UO126; rabbit anti-mouse antibody and vitronectin were from Sigma Chemical Co. (St. Louis, MO). Fibronectin was from Seromed Biochrom (Berlin, Germany).

Cell cultures. Human androgen-independent DU 145 and PC3 prostate cancer cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were routinely grown in RPMI medium (Seromed Biochrom) supplemented with fetal bovine serum (FBS; Gibco, Paisley, Scotland, UK) (5% for DU 145 and 10% for PC3 cells), glutamine (1 mM) and antibiotics (100 U/ml penicillin G sodium, 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate) in a humidified atmosphere of 5% $\text{CO}_2/95\%$ air.

Chemomigration assay. Cell migration was analyzed by means of chemomigration assays, using a 48-well Boyden's chamber (8- μm pore size) and according to the manufacturer's instructions (Neuroprobe, Cabin John, MD), as previously described (23). After the specific treatments (see below), subconfluent DU 145 cells were harvested and cell suspensions (1.5×10^5 cells/50 μl) were placed in the open bottom wells of the upper compartment of the chamber. The chemoattractant (20% FBS) was placed in the lower compartment of the chamber. The chamber was then kept for 4 h in the cell culture incubator. After that, the cells that had migrated through the pores and had adhered to the underside of the membrane were fixed, stained (Diff-Quick kit; DADE, Dudingon, CH) and mounted onto glass slides. For quantitative analysis, six random objective fields of stained cells were counted for each well (8 wells/experimental group) and the mean number of migrated cells/ mm^2 was calculated.

Experiment 1: To evaluate the role of the IGF-IR in the control of prostate cancer cell migration, DU 145 cells, in complete medium (FBS supplemented), were treated with the IGF-IR $\alpha\text{IR}3$ monoclonal antibody (1 $\mu\text{g}/\text{ml}$) for 1 h, before being harvested and seeded in the wells for the migration assay.

Experiment 2: To confirm the effect of IGF-I on cell motility, DU 145 cells, in serum-free medium, were treated with IGF-I (10 nM) for 24 h. During the last hour of treatment, the IGF-IR antibody (1 $\mu\text{g}/\text{ml}$) was added to the incubation medium. Cells were then harvested and seeded in the wells for the assay.

The specific pro-migratory effect of IGF-I on DU 145 prostate cancer cells was further investigated in another androgen-independent prostate cancer cell line (PC3). These experiments were performed as described above for DU 145 cells (same dose of IGF-I or IGF-IR antibody, same length of treatment, etc.).

Experiment 3: To investigate the possible intracellular pathways involved in the activity of IGF-I, DU 145 cells, in serum-free medium, were incubated for 45 min with the PI3-K inhibitor, LY294002 (20 μM), before treatment with IGF-I (10 nM) for 30 min. Cells were then harvested and seeded in the wells for the assay. A similar experiment was performed using the MAP kinase inhibitor, UO126 (10 $\mu\text{g}/\text{ml}$).

In each of these assays, triplicate wells were performed, and the assay was repeated three times. The data were analyzed according to Bonferroni's test after one-way ANOVA.

Scanning electron microscopy. For scanning electron microscopy, fibronectin-coated coverslips were incubated in serum-free medium with or without IGF-I (10 nM) for 30 min. The cells were washed in PBS for 10 min, fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and then washed in the same buffer. Coverslips were post-fixed in aqueous 4% OsO_4 for 20 min, washed in distilled water for 10 min, immersed in aqueous 1% tannic acid for 20 min and washed in distilled water for 10 min. This procedure was repeated 3 times at room temperature. Cells were dehydrated in alcohol, immersed in isoamylacetate and dried using the critical-point method. Coverslips were sputter-coated with gold/palladium and observed under a scanning electron microscope (Autoscan Siemens). This experiment was repeated two times for two different preparations.

Haptotactic assay. Haptotactic assays were performed in a Boyden's chamber, in which the lower surface of the membrane had been precoated with fibronectin (12 $\mu\text{g}/\text{ml}$) [one of the proteins of the extracellular matrix to which $\alpha\beta3$ integrin binds (21)]. The assay was then performed as described above.

Experiment 1: To clarify the role of the $\alpha\beta3$ integrin in the migration of prostate cancer cells towards fibronectin, DU 145 cells were plated at a density of 5×10^5 cells/plate and cultured, in complete medium, either in the absence or in the presence of the function blocking $\alpha\beta3$ antibody (1 $\mu\text{g}/\text{ml}$) for 1 h. Cells were then harvested and seeded in the wells of the Boyden's chamber.

Experiment 2: To verify whether the $\alpha\beta3$ integrin mediates the activity of IGF-I on prostate cancer cell motility towards fibronectin, DU 145 cells, in serum-free media, were

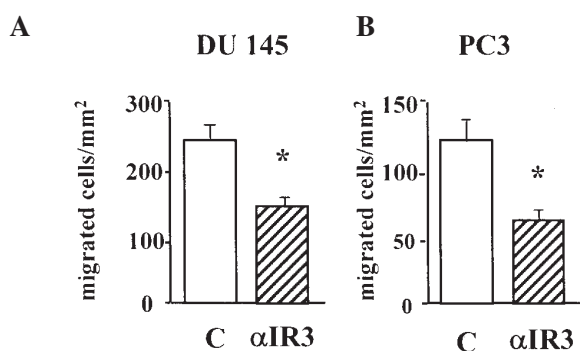


Figure 1. Effect of the neutralizing antibody, anti-IGF-IR α IR3, on the motility of androgen-independent prostate cancer cells. DU 145 and PC3 cells, cultured in complete medium (i.e. supplemented with 5 and 10% FBS, respectively), were treated with α IR3 (1 μ g/ml) for 1 h before being seeded in the wells of a Boyden's chamber. The chemomigration assay was then performed as outlined in Materials and methods. C, controls. Results are expressed as the mean number of migrated cells/mm² \pm SE. *P<0.05 vs. C.

stimulated with IGF-I (10 nM) for 24 h. The function blocking antibody against α v β 3 (1 μ g/ml) was added to the culture medium during the last hour before the haptotactic assay.

In each of these assays, triplicate wells were performed, and the assay was repeated three times. The data were analyzed according to Bonferroni's test after one-way ANOVA.

Immunofluorescence analysis. The effects of IGF-I on actin and α v β 3 integrin immunolocalization were evaluated by immunofluorescence using standard techniques. DU 145 cells were grown on 22-mm glass coverslips and treated, in serum-free conditions, with IGF-I (10 nM) for 30 min. To visualize filamentous actin (F-actin), cells were fixed with 3.7% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 1 min and stained with fluorescein isothiocyanate (FITC)-phalloidin (0.4 μ g/ml) (Sigma Chemical Co.).

For visualization of the effects of IGF-I on α v β 3 integrin expression, cells were incubated with LY294002 (20 μ M, 45 min) before treatment with IGF-I (10 nM, 30 min). Paraformaldehyde-fixed cells were incubated with an unlabeled anti- α v β 3 primary antibody, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody. Labeled cells were examined under a Zeiss Axiovert 200 microscope with a 63x/1.4 objective lens. Images were acquired using the Metavue program and analyzed using Adobe Photoshop 6.0. Each staining was repeated three times for three different preparations for each group.

Biothynylation and immunoprecipitation of the α v β 3 integrin. Experiments were performed as previously described (23). To clarify whether IGF-I could affect the levels of α v β 3 protein, DU 145 cells, grown in serum-free medium, were treated with IGF-I (10 nM), for 24 h. The cells were then washed five times with Hank's buffer and incubated with 2 ml of Hank's buffer supplemented with 500 μ g/ml sulfocinnimidyl hexanoate-conjugated biotin (NHS-LC-Biotin; Pierce Biotechnology Inc., Rockford, IL) to label cell surface proteins for 30 min at 4°C. Cells were washed three times and lysed in 0.5 ml RIPA buffer (0.05 M Tris.HCl pH 7.7, 0.15 NaCl,

0.8% SDS, 10 mM EDTA, 100 μ M NaVO₄, 50 mM NaF, 0.3 mM PMSF, 5 mM iodoacetic acid) containing leupeptin and aprotinin (1 μ g/ml). The extracts were centrifuged to remove insoluble material. Protein contents were determined using the BCA method. Equal amounts of proteins (0.5 mg) for each experimental sample were incubated overnight at 4°C with the specific antibodies (mouse anti- α v β 3, 2 μ l; and rabbit anti-mouse, 10 μ l). The antibody-integrin complexes were incubated for another 1 h with protein A-Sepharose (Pharmacia, Uppsala, Sweden), washed three times in RIPA buffer and three times in purified water. Proteins were separated by 6% SDS-PAGE, electrotransferred to nitrocellulose, incubated with peroxidase-conjugated streptavidin and visualized by chemiluminescence (SuperSignal Chemiluminescence Detection System; Pierce Biotechnology Inc.).

Results

IGF-I stimulates prostate cancer cell migration through the activation of IGF-IR. These studies were performed to clarify whether the IGF-I-induced activation of IGF-IR might affect the migratory properties of the androgen-independent prostate cancer cell lines, DU 145 and PC3. DU 145 cells, grown in complete medium (i.e. FBS-supplemented and, therefore, in the presence of growth factors), were treated for 1 h with a specific antibody against IGF-IR (α IR3) before being seeded in the upper compartment of a Boyden's chamber (chemotactic assay). Fig. 1A shows that control DU 145 cells actively migrated towards the chemoattractant (20% FBS); although, when the activity of IGF-IR was blocked by the specific antibody, the pro-migratory effect of serum was significantly reduced (Fig. 1A). Similar results were obtained using another androgen-independent prostate cancer cell line (PC3) (Fig. 1B). To confirm the specificity of the antibody, we showed that the presence of an aspecific antibody (human IgG) did not affect the migration of either prostate cancer cell line (data not shown).

These results suggest that IGF-I might be involved in the control of the motile behavior of prostate cancer cells. To validate these observations, DU 145 cells, grown in the absence of serum, were treated with IGF-I (10 nM), either alone or in the presence of the function blocking antibody raised against IGF-IR. We observed that IGF-I significantly increased the migratory capacity of DU 145 cells; this effect was completely blocked by the simultaneous treatment of the cells with the IGF-IR antibody (Fig. 2A). When given alone, α IR3 did not affect the motility of the cells. Identical results were obtained with PC3 cells (Fig. 2B).

Collectively, these data demonstrate that IGF-I through the activation of its receptor, IGF-IR, exerts a pro-migratory activity on androgen-independent prostate cancer cells.

IGF-I induces lamellipodia formation and actin cytoskeleton rearrangements. The metastatic behavior of tumor cells is usually associated with change in cell morphology, actin polymerization and rearrangement of actin filaments. To correlate the data obtained from the migration assays with cell morphology, scanning electron microscopy was performed. Fig. 3B indicates the formation of lamellipodia in IGF-I-treated DU 145 cells with respect to controls (Fig. 3A). This

macrograph also reveals that the bodies of IGF-I-treated cells did not adhere to the substratum.

The effects of IGF-I on cytoskeleton organization were analyzed by immunofluorescent staining using TRITC-

phalloidin, which shows high affinity for the actin cytoskeleton. Fig. 4 shows that, in DU 145 cells, IGF-I induced an arrangement of the actin cytoskeleton in stress fibers and an increased intensity of actin immunofluorescence in peripheral areas below the cell membrane (Fig. 4).

The PI3-K/Akt signaling pathway mediates the pro-migratory activity of IGF-I. The activation of the PI3-K/Akt and MAP kinase signaling pathways by IGF-I has been reported to be deeply involved in the growth of several tumors, including prostate cancer (8,15,24,25). In DU 145 cells, in particular, IGF-I has been previously demonstrated to stimulate Akt and ERK1/2 phosphorylation (25,26). Experiments have been performed to clarify whether these pathways might mediate the pro-migratory activity of IGF-I in DU 145 androgen-independent prostate cancer cells. Therefore, we measured the effects of the PI3-K inhibitor, LY294002, and of the MAP kinase inhibitor, UO126, on IGF-I-induced DU 145 cell migration. Fig. 5A shows that, as expected, IGF-I stimulated the migratory activity of the cells while LY294002, when given alone, did not modify their motile behavior. Pretreatment of DU 145 cells with LY294002 completely counteracted the pro-migratory effect of the growth factor. On the contrary, Fig. 5B shows that UO126 was not able to affect the IGF-I-induced migration of DU 145 cells. Thus, IGF-I-stimulated motility of prostate cancer cells seems to depend on the activation of the PI3-K/Akt signaling pathway.

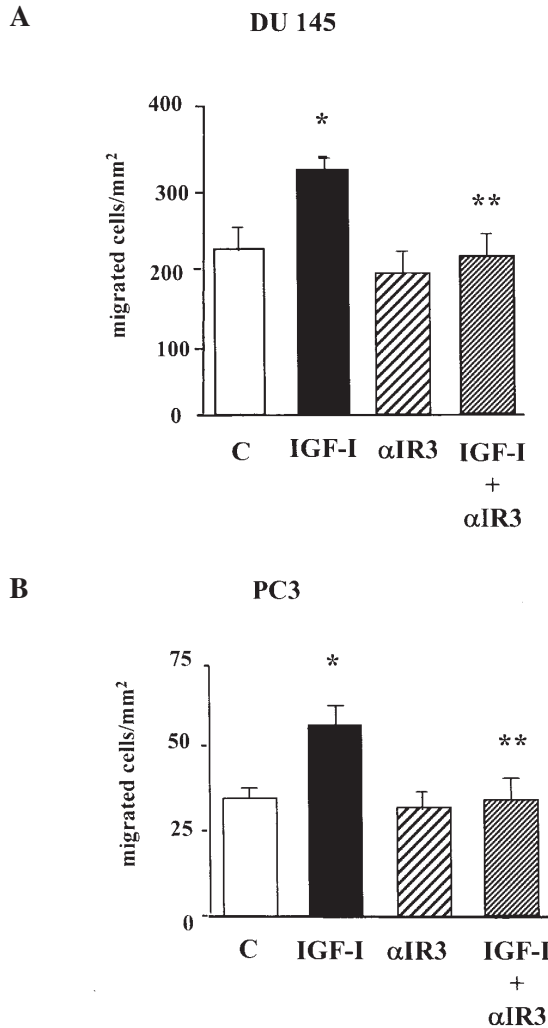


Figure 2. Effect of IGF-I, either in the absence or presence of the neutralizing antibody, anti-IGF-IR α IR3, on the motility of androgen-independent prostate cancer cells (DU 145 and PC3). Cells, grown in the absence of serum, were treated with IGF-I alone (10 nM, 24 h), with α IR3 alone (1 μ g/ml, 1 h), or with IGF-I (24 h) in the presence of α IR3 (during the last hour of incubation). The chemomigration assay was then performed as outlined in Materials and methods. Results are expressed as the mean number of migrated cells/mm² \pm SE. C, controls. * P <0.05 vs. C; ** P <0.05 vs. IGF-I.

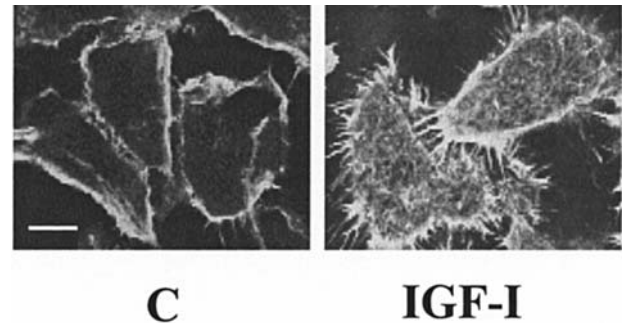


Figure 4. Immunofluorescence staining of F-actin showing the effects of IGF-I on the actin cytoskeleton organization of DU 145 cells. IGF-I (10 nM) induces a change in cell morphology with formation of lamellipodia and a redistribution of the actin cytoskeleton with an increased intensity of the immunofluorescence in peripheral areas below the cell membrane and along the leading edge of the lamellipodia. Scale bar, 16 μ m.

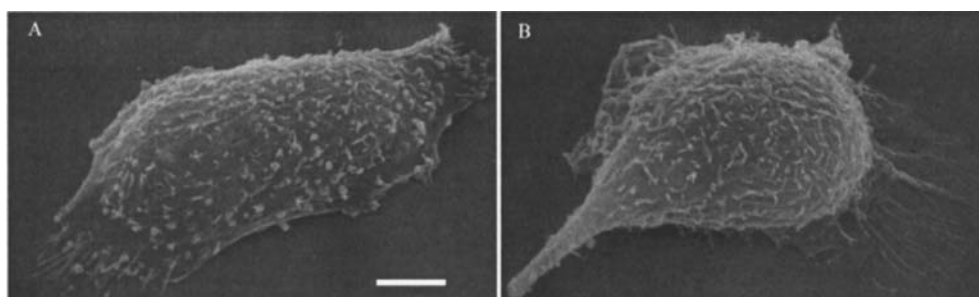


Figure 3. Scanning electron microscopy of serum-starved DU 145 cells, untreated (A) and treated with IGF-I (10 nM) (B). In DU 145 cells, IGF-I induced the formation and extension of lamellipodia. Scale bar, 10 μ m.

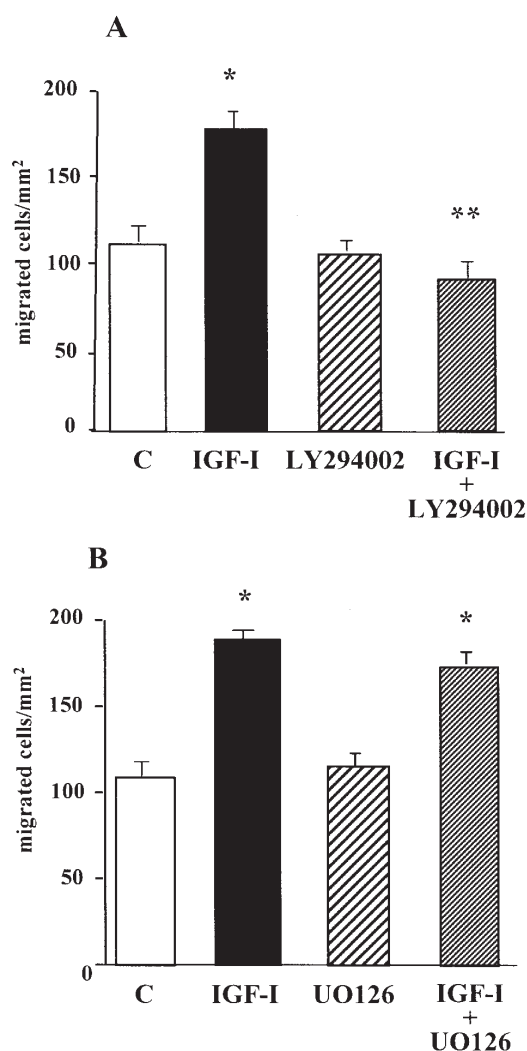


Figure 5. Involvement of the PI3-K/Akt and of the MAP kinase signaling pathways in the pro-motility activity of IGF-I on DU 145 cells. DU 145 cells, in serum-free medium, were incubated with LY294002 (20 μM) (A) or with UO126 (10 μM) (B) for 45 min before treatment with IGF-I (10 nM) for 30 min. The chemotaxis assay was then performed as outlined in Materials and methods. Results are expressed as the mean number of migrated cells/mm² ± SE. C, controls. *P<0.05 vs. C; **P<0.05 vs. IGF-I.

Anti-αvβ3 integrin antibody blocks the IGF-I-induced migration of prostate cancer cells. The αvβ3 integrin dimer, highly expressed in androgen-independent prostate cancer cells, has been suggested to be involved in the processes of prostate cancer cell motility (27). These studies were performed to clarify whether the αvβ3 integrin might play some role in IGF-I-induced DU 145 cell migration towards fibronectin. These experiments were performed by means of a haptotactic assay, which allows the determination of the migration of the cells towards a specific extracellular matrix ligand (fibronectin) for the integrin of interest (αvβ3). In preliminary experiments, we showed that pre-treatment of the cells (30 min) cultured in complete medium with a monoclonal antibody (function blocking) against αvβ3 significantly reduced the ability of the cells to move towards fibronectin (Fig. 6A), confirming the crucial role of this integrin in the motility behavior of prostate cancer cells. The motility of the cells was not affected by the presence of an aspecific antibody (human IgG) (data not shown). By using the same haptotactic assay, we further

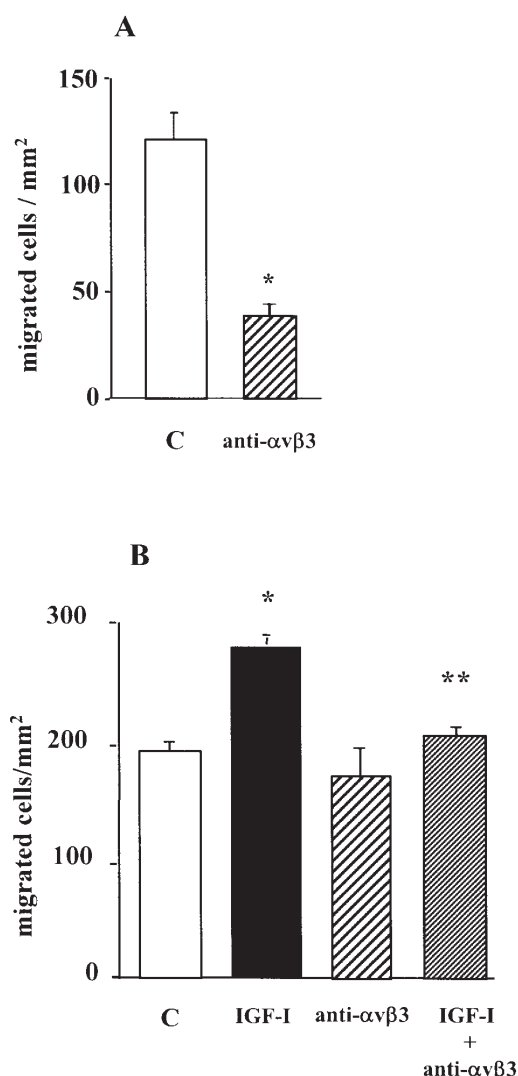


Figure 6. Role of the αvβ3 integrin in the pro-migratory activity of IGF-I on androgen-independent prostate cancer cells. These experiments have been performed by means of a haptotactic assay, as outlined in Materials and methods. (A) Effect of a function blocking antibody against αvβ3 (1 μg/ml, for 1 h) on the motility of DU 145 cells, grown in complete medium, towards fibronectin. (B) Effect of IGF-I, either in the absence or in the presence of the antibody anti-αvβ3, on the motility of DU 145 cells towards fibronectin. Cells grown in the absence of serum were stimulated with IGF-I alone (10 nM, 24 h), with anti-αvβ3 (1 μg/ml, 1 h), or with IGF-I (24 h) in the presence of anti-αvβ3 (during the last hour of incubation). Results are expressed as the mean number of migrated cells/mm² ± SE. *P<0.05 vs. controls (C); **P<0.05 vs. IGF-I.

demonstrated that the migration of DU 145 cells cultured in serum-free medium was significantly stimulated by IGF-I; the function blocking antibody against αvβ3 completely reversed the action of IGF-I (Fig. 6B). Anti-αvβ3, when given alone, did not affect cell migration (Fig. 6B).

IGF-I increases αvβ3 integrin immunofluorescence through PI3-K/Akt. The experiments described above indicate that the activity of αvβ3 is crucial for IGF-I to exert its pro-migratory effect. We then tested whether the growth factor might affect also the expression and/or the cellular distribution of the integrin in DU 145 cells. Using immunofluorescence, we showed, after 30 min of treatment with the growth factor, an increased intensity of the fluorescence corresponding to αvβ3,

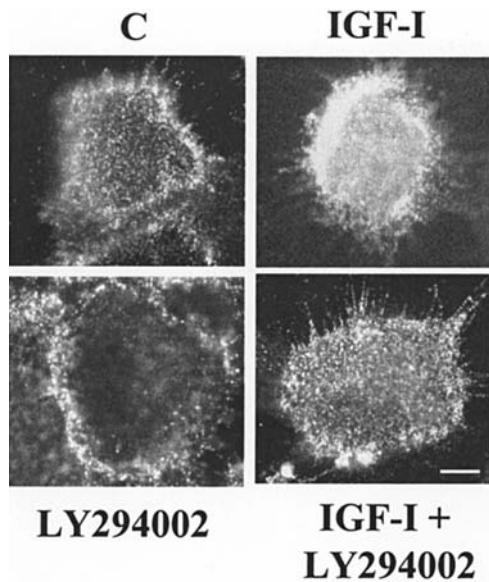


Figure 7. Effect of IGF-I on $\alpha v\beta 3$ immunofluorescence. DU 145 cells were treated with LY294002 (20 μM , 45 min) before treatment with IGF-I (10 nM) for 30 min. Immunofluorescence analysis reveals, in IGF-I-treated cells, an increased intensity of $\alpha v\beta 3$ staining which is mainly localized at the level of the cell membranes; the blockade of PI3-K activity counteracts the increased intensity of $\alpha v\beta 3$ staining induced by IGF-I. C, controls. Scale bar, 16 μm .

which is mainly localized at the level of the cell membrane (Fig. 7). The PI3-K inhibitor, LY294002, clearly antagonized this effect (Fig. 7). These data demonstrate that the pro-migratory activity of IGF-I on androgen-independent prostate cancer cells is mediated by an increased expression of $\alpha v\beta 3$ fluorescence at the level of the cell membrane, and this effect is mediated by the PI3-K/Akt pathway.

IGF-I increases $\alpha v\beta 3$ protein expression. Experiments were performed to clarify whether IGF-I might affect also the expression of $\alpha v\beta 3$ at the protein level. A 24-h period of treatment with the growth factor was selected in order to detect possible modifications of protein synthesis. Cells were treated with the growth factor for 24 h. By biotinylation, immunoprecipitation and Western blot analysis, we demonstrated that the treatment of the cells with IGF-I increased the expression of $\alpha v\beta 3$ protein (Fig. 8); interestingly, this effect was not modified by a co-treatment of the cells with LY294002 (data not shown). These data indicate that the PI3-K/Akt pathway might not mediate the effects of IGF-I on the expression of the $\alpha v\beta 3$ protein.

It is known that Akt phosphorylation represents a very rapid event in the action of IGF-I. Therefore, it is possible that the activation of this kinase might play a crucial role in triggering the early molecular events of the migratory response of the cells (such as $\alpha v\beta 3$ cellular localization) to the growth factor; delayed effects, such as regulation of $\alpha v\beta 3$ protein expression, might involve different mediators placed downstream in the IGF-I signaling cascade.

Discussion

The data reported in this paper represent the first demonstration that IGF-I, through the activation of its specific

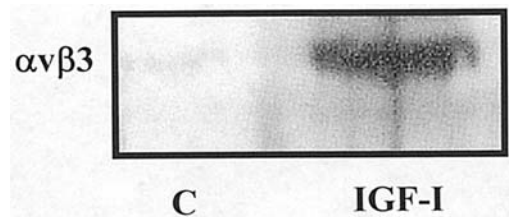


Figure 8. Effect of IGF-I on $\alpha v\beta 3$ protein expression. DU 145 cells were treated with IGF-I (10 nM) for 24 h; protein levels were evaluated by Western blotting after protein biotinylation and immunoprecipitation. One of three experiments is reported.

receptor, IGF-IR, promotes the motility of androgen-independent prostate cancer cells (both DU 145 and PC3). The increased migratory ability of the cells is accompanied by the formation of lamellipodia, extending in multiple directions and by actin cytoskeletal reorganization. Since IGF-I-induced migration is completely antagonized by the presence of a specific PI3-K inhibitor (but not by the presence of a MAP kinase inhibitor), we conclude that the pro-migratory effect of the growth factor specifically requires the activation of the PI3-K/Akt signaling pathway.

In agreement with our observations, Grzmil and coworkers (28) recently reported that the blockade of IGF-IR expression in androgen-independent prostate cancer PC3 cells results in a significant suppression of the invasive behavior of the cells (ability of the cells to invade a Matrigel preparation), which is accompanied by up-regulation of IGF binding protein-3 and suppression of matrix metalloproteinase-2 (MMP-2) expression.

The crucial role of IGF-I, both serum-derived and locally produced, in the control of prostate cancer cell proliferation has been widely demonstrated in our laboratory (13) and others (10-12,14). The data here demonstrate that IGF-I promotes not only the growth but also the metastatic (migratory and invasive) behavior of prostate cancer cells.

The pro-migratory activity of IGF-I in prostate cancer is in line with previous observations obtained in other types of tumors (24,29). IGF-I has been shown to increase cell motility in breast cancer cells (30,31); this effect is accompanied by the formation of motile actin microspikes at the level of cell-cell contact and disorganization of adherens junctions (30). Moreover, in highly metastatic breast cancer cells, the transfection of the dominant negative forms of IGF-IR abrogated cellular motility and adhesion to extracellular matrix proteins (laminin and collagen), and inhibited IGF-I-stimulated invasion through collagen (32,33). When injected into the mammary fat pad, breast cancer cells expressing the dominant negative IGF-IR did not give rise to metastases, especially to the lungs (33). IGF-I has been found to stimulate both the migratory and invasive behavior of colon cancer cells, either *in vitro* or *in vivo* (when grown in nude mice); this effect is accompanied by increased activity of the urokinase plasminogen activator (uPA)/uPAR system, a key mediator of tumor cell metastatic behavior (34-36). In lung carcinoma cell lines, IGF-I has been demonstrated to increase the invasive and motile capabilities of the cells and this action was accompanied by an up-regulation of the activity of matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9

(37-39). Finally, Tai and coworkers (40) have shown that IGF-I induces both adhesion and migration in myeloma cells, through the activation of the $\beta 1$ integrin.

In this study, we investigated whether cell adhesion molecules, such as integrins, might be involved in the promigratory activity of IGF-I on prostate cancer cells. We have first shown that $\alpha \beta 3$ integrin is deeply involved in the control of the motility of DU 145 cells, since a function blocking antibody against this integrin dimer completely suppressed the migration of the cells towards fibronectin, one of its extracellular matrix ligands. The level of expression of the $\alpha \beta 3$ integrin has been previously shown to positively correlate with the grade of malignancy of prostate cancer. This integrin is not expressed in androgen-dependent, poorly aggressive, LNCaP cells, while it is expressed at high levels in androgen-independent, strongly metastatic, PC3 cells (22). Exogenous expression of $\alpha \beta 3$ in LNCaP cells results in increased cell adhesion to and migration on vitronectin (22), in an activation of the PI3-K/Akt pathway (41) and in increased levels of cdc2 protein and kinase activity (27). We then demonstrated that a function blocking antibody against $\alpha \beta 3$ completely abrogated the IGF-I-induced migration of DU 145 cells in a haptotactic assay. IGF-I also increased $\alpha \beta 3$ immunofluorescence, which localized mainly at the membrane level, and this effect was counteracted by the blockade of the PI3-K activity. Collectively, these data indicate that IGF-I promotes the motile behavior of androgen-independent prostate cancer cells through $\alpha \beta 3$ integrin activation/expression and that this effect appears to be mediated by the PI3-K/Akt signaling pathway.

Our observations are in line with recent studies on non-malignant cells; the $\alpha \beta 3$ integrin dimer has been shown to be required for IGF-I to stimulate the proliferation and migration of smooth muscle cells (42), extravillous trophoblast cells (43), and preadipocyte 3T3-L1 cells (44). In smooth muscle cells, the intracellular proteins (DOK1, SHP-2) that are recruited to the $\beta 3$ subunit of the dimer following IGF-IR stimulation, have also been identified (45-47).

The data so far available on the possible role of $\alpha \beta 3$ in the IGF-I-induced modulation of proliferation and motility in cancer cells are still scanty and, apparently, have led to divergent conclusions. Pereira and coworkers have recently reported that, in MCF-7 breast cancer cells, stable expression of $\alpha \beta 3$ abrogates the IGF-I-mediated migration of the cells through its interaction with IGF-binding protein-2 (IGFBP-2) (48). These data, supporting a negative regulatory role of $\alpha \beta 3$ in the chemotactic activity of IGF-I in breast cancer cells, are in contrast with those reported in this paper. The reason for this discrepancy is unclear; however, it might be related to the different cancer cell types analyzed. In this context it must be underlined that, according to Pereira and coworkers (48), $\alpha \beta 3$ expression seems to be down-regulated in breast tumors when compared to that in non-neoplastic breast tissue. As already reported, in prostate cancer, the expression of the integrin positively correlates with the level of malignancy of the tumor. Moreover, MCF-7 cells lack expression of $\alpha \beta 3$, which is, in contrast, highly expressed in the prostate cancer cells utilized in the present study.

In conclusion, the data reported in this paper demonstrate that: i) IGF-I promotes the motile behavior of androgen-

independent prostate cancer cells, through $\alpha \beta 3$ integrin activation/expression; ii) these effects are mediated by the PI3-K/Akt signaling pathway. These results support a role for IGF-I in the progression of prostate cancer towards the metastatic phase (in addition to its role in the growth of the tumor), and provide an additional rationale basis for the development of therapeutic strategies involving the IGF-I/IGF-IR system in the treatment of prostate cancer.

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