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, αvβ3, 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 αvβ3; b) IGF-I increases αvβ3 immunofluorescence at the level of cell membranes, and this effect is counteracted by LY294002; and c) IGF-I increases αvβ3 protein levels. Our results demonstrate that IGF-I promotes the motility of androgen-independent prostate cancer cells by modulating αvβ3 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 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-
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, αvß3, 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 αvß3 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 Peprotech EC, Ltd. (London, UK) and neutralizing anti-IGF-IR mAb dR3 was obtained from Oncogene Research Products (San Diego, CA). Mouse anti-αvß3 antibody was from Chemicon (Temecula, CA). The phosphatidylinositol3-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 μg/ml streptomycin sulphate) in a humidified atmosphere of 5% CO2/95% air.

Chemosmigration 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), sub-confluent DU 145 cells were harvested and cell suspensions (1.5x10⁵ 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, Dudingen, 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/mm² 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 dR3 monoclonal antibody (1 μg/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 μg/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 μg/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% OsO4 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 isomylacetate 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 μg/ml) [one of the proteins of the extracellular matrix to which αvß3 integrin binds (21)]. The assay was then performed as described above.

Experiment 1: To clarify the role of the αvß3 integrin in the migration of prostate cancer cells towards fibronectin, DU 145 cells were plated at a density of 5x10⁵ cells/plate and cultured, in complete medium, either in the absence or in the presence of the function blocking αvß3 antibody (1 μg/ml) for 1 h. Cells were then harvested and seeded in the wells of the Boyden’s chamber.

Experiment 2: To verify whether the αvß3 integrin mediates the activity of IGF-I on prostate cancer cell motility towards fibronectin, DU 145 cells, in serum-free media, were
Experiments were performed as previously described (23). For three different preparations for each group, Adobe Photoshop 6.0. Each staining was repeated three times acquired using the Metavue program and analyzed using 200 microscope with a 63x/1.4 objective lens. Images were body. Labeled cells were examined under a Zeiss Axiovert cyanate (FITC)-conjugated goat anti-mouse secondary anti-

...isothiocyanate (FITC)-phalloidin (0.4 μg/ml) (Sigma Chemical Co.). For visualization of the effects of IGF-I on αvβ3 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 μg/ml) (Sigma Chemical Co.).

For visualization of the effects of IGF-I on αvβ3 integrin expression, cells were incubated with LY294002 (20 μM, 45 min) before treatment with IGF-I (10 nM, 30 min). Paraformaldehyde-fixed cells were incubated with an unlabeled anti-αvβ3 primary antibody, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody. Labeled cells were examined under a Zeiss Axiobase 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.

Biothyllination and immunoprecipitation of the αvβ3 integrin. Experiments were performed as previously described (23). To clarify whether IGF-I could affect the levels of αvβ3 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 μg/ml sulfoconimidyl hexanote-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 μM NaVO₄, 50 mM NaF, 0.3 mM PMSF, 5 mM iodoacetic acid) containing leupeptin and aprotinin (1 μg/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-αvβ3, 2 μl; and rabbit anti-mouse, 10 μl). 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 (αIR3) 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, αIR3 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).
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-phallolidin, 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 αIR3, 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 αIR3 alone (1 μg/ml, 1 h), or with IGF-I (24 h) in the presence of αIR3 (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 ± SE. C, controls. *P<0.05 vs. C; **P<0.05 vs. IGF-I.

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 μm.

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 μm.
Anti-\(\alpha\beta^3\) integrin antibody blocks the IGF-I-induced migration of prostate cancer cells. The \(\alpha\beta^3\) 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 \(\alpha\beta^3\) 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 (\(\alpha\beta^3\)). In preliminary experiments, we showed that pre-treatment of the cells (30 min) cultured in complete medium with a monoclonal antibody (function blocking) against \(\alpha\beta^3\) 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 demonstrated that the migration of DU 145 cells cultured in serum-free medium was significantly stimulated by IGF-I; the function blocking antibody against \(\alpha\beta^3\) completely reversed the action of IGF-I (Fig. 6B). Anti-\(\alpha\beta^3\), when given alone, did not affect cell migration (Fig. 6B).

**IGF-I increases \(\alpha\beta^3\) integrin immunofluorescence through PI3-K/Akt.** The experiments described above indicate that the activity of \(\alpha\beta^3\) 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 \(\alpha\beta^3\),
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IGF-I increases αvβ3 protein expression. Experiments were performed to clarify whether IGF-I might affect also the expression of αvβ3 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 biothynilation, immunoprecipitation and Western blot analysis, we demonstrated that the treatment of the cells with IGF-I increased the expression of αvβ3 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 αvβ3 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 αvβ3 cellular localization) to the growth factor; delayed effects, such as regulation of αvβ3 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 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.

Figure 7. Effect of IGF-I on αvβ3 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 αvβ3 staining which is mainly localized at the level of the cell membranes; the blockade of PI3-K activity counteracts the increased intensity of αvβ3 staining induced by IGF-I. C, controls. Scale bar, 16 μm.

Figure 8. Effect of IGF-I on αvβ3 protein expression. DU 145 cells were treated with IGF-I (10 nM) for 24 h; protein levels were evaluated by Western blotting after protein biothynilation and immunoprecipitation. One of three experiments is reported.
haptotactic assay. IGF-I also increased 
must be underlined that, according to Pereira and coworkers 
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the β3 subunit of the dimer following IGF-IR stimulation, 
the intracellular proteins (DOK1, SHP-2) that are recruited to 
and preadipocyte 3T3-L1 cells (44). In smooth muscle cells, 
and that: i) IGF-I promotes the motile behavior of androgen-
expression of 
independent prostate cancer cells, through αvβ3 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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