Novel mechanism of IGF-binding protein-3 action on prostate cancer cells: inhibition of proliferation, adhesion, and motility

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Abstract

IGF-binding protein-3 (IGFBP-3) is a modulator of the IGF-signaling pathway and was described as an anti-cancer agent in prostate cancer. The molecular mechanisms underlying these effects remained, however, largely undefined. We analyzed the influence of recombinant IGFBP-3 on cell proliferation of PC3, Du145, and LNCaP prostate cancer cells. As expected, IGFBP-3 inhibited IGF-stimulated cell proliferation by blocking IGF-mediated proliferation signals, but we observed an IGF-independent inhibitory effect of IGFBP-3 on prostate cancer cell proliferation in long-term cultures. We further investigated the influence of IGFBP-3 on adhesion, motility, and invasion of prostate cancer cells using adhesion assays, live-cell imaging techniques, and matrigel invasion measurements. There was a clear inhibitory effect of IGFBP-3 on tumor cell adhesion to extracellular matrix components in the presence and absence of IGF, whereas cell–cell adhesion was not affected. The same inhibitory effect of IGFBP-3 was determined on cell motility when real-time cell movements were followed. In addition, IGFBP-3 was able to inhibit tumor cell invasion through matrigel. In summary, we show that IGFBP-3 inhibits proliferation, adhesion, migration, and invasion processes of prostate tumor cells. These newly described mechanisms of IGFBP-3 can be of importance for tumor progression and support a role of IGFBP-3 in therapeutic settings.

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Introduction

Prostate cancer is now the most frequently diagnosed cancer among men in Europe and the United States as well as a common cause of cancer death all over the world (Parkin *et al.* 2005, Jemal *et al.* 2008). A special feature of prostate cancer is that a latent form is very common and most cases of prostate cancer grow very slowly. Many men die with, rather than from, prostate cancer and never reach the stages of advanced prostate cancer (Selman 2000). However, progression of prostate cancer to metastatic cancer, as it occurs in approximately one-third of all cases, leads to the development of an aggressive disease that is lethal for the patients. Large effort is, therefore, placed in understanding the molecular mechanisms driving prostate cancer progression in order to enable the

development of effective therapeutic strategies targeting tumor progression and metastasis formation.

The progression of prostate cancer to metastatic cancer is a complex multi-step process that starts with the transformation of normal cells and continues in terms of local tumor growth, tissue extravasation of transformed tumor cells, invasion of adjacent tissue, and travel to lymph nodes or distant organs, mainly bones, where metastatic colonies are founded (Hanahan & Weinberg 2000). Tumor cell progression is dependent on the epithelial as well as the stromal compartment, which is able to influence proliferation, adhesion, migration, and the metastatic behavior of tumor cells due to modification of the extracellular matrix and growth factor production (Rowley 1998, Tuxhorn *et al.* 2001, Sung & Chung 2002).

One protein family shown to play a key role in several of these processes is the insulin-like growth factor (IGF) family. There is abundant *in vitro* animal and epidemiologic evidence to suggest that the IGF family is a multi-component network of molecules, which is involved in the regulation of both physiological and pathological growth processes in the prostate (Gennigens *et al.* 2006). IGFs stimulate cell proliferation by binding to high-affinity IGF-I receptors and activating the receptor tyrosine kinase. The activities of the growth factors IGF-I and IGF-II are modulated by a family of IGF-binding proteins (IGFBPs). The IGFBPs stabilize the IGFs due to the formation of IGF/IGFBP complexes. This leads to the sequestration of IGFs from their cell surface receptors and a resultant inhibition of downstream signaling events (Jones & Clemmons 1995, Hwa *et al.* 1999). IGFBP-3 is the most abundant IGFBP in the blood and was also described as having ‘IGF-independent’ functions since IGFBP-3 was shown to exhibit pro-apoptotic (Rajah *et al.* 1997, Butt *et al.* 2000, Williams *et al.* 2007) and anti-proliferative functions (Oh *et al.* 1995, Boyle *et al.* 2001, Silha *et al.* 2006, Alami *et al.* 2008), and was described to be a suppressor of angiogenesis (Liu *et al.* 2007). Various studies indicated that IGFBP-3 also acts as an anti-cancer molecule in prostate cancer (Rajah *et al.* 1997, Bhattacharyya *et al.* 2006, Kojima *et al.* 2006, Liu *et al.* 2007, Silha *et al.* 2006, Peng *et al.* 2007). Furthermore, serum IGFBP-3 may also modulate prostate carcinogenesis, although case–control studies correlating IGFBP-3 concentrations in the blood circulation and prostate cancer risk are contradictory (Renehan *et al.* 2004).

In addition to circulating IGFBP-3, which originates mainly from the liver, there is also a significant local synthesis of the peptide in the prostate. We have previously shown that the prostatic stroma produces and secretes IGFBP-3 (Massoner *et al.* 2008). Stromal elements are important mediators of tumor development, growth, and progression. Stromal factors can promote or inhibit tumor growth as well as tumor progression, and they are able to influence whether local tumors remain organ confined or progress to metastatic disease (Rowley 1998, Tuxhorn *et al.* 2001, Sung & Chung 2002). In this study, we addressed the question how IGFBP-3, when present in the cellular microenvironment, exerts its functions as an anti-cancer molecule on prostate cancer cells in terms of growth, adhesion, migration, and invasion. We describe novel mechanisms of IGFBP-3, showing that IGFBP-3 is able to influence adhesion, motility, and – in some cellular cancer models – also invasion of

prostate cancer cells, and we confirmed an inhibitory effect of IGFBP-3 on prostate cancer cell proliferation. Our data provide new molecular mechanisms of IGFBP-3 acting as an anti-tumor protein in prostate cancer and support herewith its use in therapeutic settings.

Material and methods

Cell culture

LNCaP were grown in RPMI 1640 in the presence of 10% FCS, 2 mM glutamax (Invitrogen), and antibiotics. PC3 and Du145 cells were established to grow without serum in a defined medium composed of RPMI 1640 containing 0.5 mg/ml albumax (Invitrogen), 2 mM glutamax (Invitrogen), 1% chemically defined lipid concentrate (Invitrogen), 1% vitamin solution (Sigma), 1% nonessential amino acid solution (PAA Laboratories, Pasching, Austria), 5 mg/l insulin (Invitrogen), 3.5 µg/l sodium selenite (Invitrogen), 2.75 mg/l transferrin (Invitrogen), 100 nM hydrocortisone, and antibiotics. To adapt the cells for growing in serum-free medium, the serum concentration was stepwise reduced from 10% down to 3, 1, and 0.2% until finally reaching 0%. Cell passaging was performed by using a soybean trypsin inhibitor (Sigma) for trypsin inactivation. For proliferation, adhesion, motility, and invasion assays, the insulin concentration in the serum-free culture medium was decreased to 5 µg/l. Human bone marrow endothelial cells (HBMEC-60) were cultured in endothelial cell culture medium in culture flasks coated with fibronectin as previously described (Rood *et al.* 2000).

Substances and treatments

IGFBP-3 was produced and purified as described below. In addition, IGFBP-3 was purchased from a commercial supplier (R&D Systems, Minneapolis, MN, USA) and used as a control. About 500 ng/ml IGFBP-3, 10 ng/ml IGF-I (Sigma), 400 µM cycloheximide (Sigma), 60 µM etoposide (Sigma), 500 ng/ml heparin-binding protein (HBP, azurocidin, CAP37; Sigma), 500 ng/ml IGFBP-5 (kindly provided by Dr Doppler (Jurgeit *et al.* 2007)), 8 ng/ml insulin (Sigma), 10 µg/ml MAB391 (R&D Systems), 2 µM chlorpromazine (Sigma), 50 µM amantadine (Sigma), 50 µg/ml nystatin (Sigma), 10 mM β-cyclodextrin (Sigma), and 50 µg/ml genistein (Sigma) were used for the experiments, unless otherwise stated.

Purification of native IGFBP-3 by affinity chromatography

Cell culture supernatant from U2OS cells overexpressing IGFBP-3 and from mock-transfected cells respectively was harvested. Four protease inhibitor tablets (Complete, Roche) and 1/9 volume of 500 mM sodium phosphate buffer (pH 6.5) were added to 200 ml cell culture supernatant. After filtration through a 0.45 µm bottle-top filter (Steritop, Millipore, Schwalbach, Germany), the cleared supernatant was applied to a 1 ml heparin column (HiTrap, GE Healthcare, Vienna, Austria) equilibrated with 50 mM sodium phosphate buffer (pH 6.5). Protein bound to the column was eluted by applying a salt gradient ranging from 50 mM to 1 M NaCl in 50 mM sodium phosphate buffer (pH 6.5) and collected in 1 ml fractions. Fractions containing IGFBP-3 were identified by Western blot. The concentration of the recombinant produced IGFBP-3 was determined using a sandwich ELISA (R&D Systems). A purification control (supernatant of mock-transfected U2OS, which was subjected to the same purification procedure as supernatant of U2OS cells overexpressing IGFBP-3) was performed for all purification charges.

Cell proliferation assay

Cells were seeded in 12-well plates at a density of 160 000 cells per well. Substances were added every 24 h, starting 24 h after seeding. Four days after seeding, when the cells reached confluence, they were washed in PBS, trypsinized, and the cell number was determined using CASY Cell Counter and Analyzer System (Schärfe System, Reutlingen, Germany). The cells were reseeded in new 12-well culture plates at a density of 160 000 cells per well in the presence of the defined substances and cultured for another 4 days, before they were harvested and counted again.

Cell apoptosis assay

Cells were seeded in 24-well plates at a density of 100 000 cells per well. They were exposed to IGF and IGFBP-3 for 72 h or treated with apoptosis-inducing substances (etoposide and cycloheximide) in the presence or absence of IGFBP-3 for 48 h. Apoptosis was measured using EnzChek Caspase-3 Assay Kit #2 (Invitrogen) according to the manufacturer's instructions. Shortly, active caspase-3 in cellular lysates of the treated cells was detected upon cleavage of a nonfluorescent substrate to a fluorescent amide mediated by DEVD-specific proteases (caspase-3 and – to some extent – caspase-7). Fluorescent signals were

measured in a fluorescent plate reader (Chameleon V, Hidex, Turku, Finland) and normalized against total protein amounts (determined using the Bradford method). In addition, we used the cellular lysates for the detection of cleaved poly(ADP-ribose) polymerase (PARP) by Western blot (see below).

Cell adhesion assay

Cells were seeded in six-well culture dishes at a density of 500 000 cells per well. After defined time points (2 and 6 h), the floating cells were removed by washing twice with PBS. The remaining adherent cells were detached from the culture surface using trypsin–EDTA, harvested, and counted using the CASY Cell Counter and Analyzer System (Schärfe System). To investigate cell adhesion to extracellular matrix components, the assay was further optimized: cellular adhesion was tested by coating culture surfaces of 96-well plates for 3 h with 100 µl/well of the following extracellular matrix components: 0.2% gelatin (Sigma); 15 µg/ml laminin (Sigma); 6 µg/ml fibronectin (Becton Dickinson, Franklin Lakes, NJ, USA); or 30 µg/ml polylysine (Sigma). Prostate tumor cells were fluorescently labeled with 4 µg/ml calcein AM (Sigma) for 11 h and washed twice in PBS prior to seeding. About 40 000 cells per well were seeded in each well of the coated 96-well plates and let adhere to the surfaces for 2 h. Subsequently, the plates were washed twice in PBS to remove all nonadherent cells. The adherent cells were lysed in water containing 2% Triton X-100, and the fluorescence intensity was measured on a Chameleon V plate reader (Hidex). The same protocol (without coating) was used to test cell adhesion when endocytosis was inhibited. Fluorescently labeled prostate cancer cells were preincubated with inhibitors of clathrin-mediated (chlorpromazine and amantadine) and caveolae-mediated (nystatin, cyclodextrin, and genistein) endocytosis (for concentrations see substances and treatments) for 30 min prior testing their adhesion in the presence and absence of IGFBP-3 according to the above described protocol.

Epithelial/endothelial interaction assay

Immortalized HBMEC-60 (Rood *et al.* 2000) were cultured on fibronectin-coated 96-well plates until confluent. Then they were starved in serum-free medium for 3 h. In the meantime, prostate tumor cells were labeled with 4 µg/ml calcein AM for 1 h, washed in PBS, resuspended in medium 199 supplemented with 0.1% BSA, and added to the endothelial cells. After different time points, the nonadherent cells were removed by washing twice in PBS.

Tumor cells that adhered to the endothelial cell layer were measured in a fluorescent plate reader (Chameleon V, Hidex).

Cell motility assay

Cells were seeded in 12-well culture dishes at a density of 160 000 cells per well and incubated under a Cell-IQ live-cell imaging platform (Chip-man Technologies, Tampere, Finland). The imaging was started when most of the cells had already adhered (~12 h after seeding). A picture was taken every 10 min using a tenfold objective. The movement of the cells was analyzed using ImageJ software version 1.38r (Rasband 1997–2007) and the special plug-in Manual Tracking (<http://rsb.info.nih.gov/ij/plugins/track/track.html>) coded by Fabrice P Cordelières, Institut Curie, Orsay (France).

Matrigel invasion assay

The invasion capacity of cancer cells was measured using BD BioCoat Tumor Invasion System (Becton Dickinson) according to the manufacturer's instructions. Shortly, the system consists of 24-well insert plates with an 8 µm pore size membrane coated with matrigel. Cells were seeded in the insert chambers and can invade to the lower side of the membrane if they are able to migrate across the matrigel layer. PC3 cells were seeded at a density of 100 000 cells per well; Du145 and LNCaP were seeded at a density of 150 000 cells per well. Serum-free-cultured Du145 and PC3 cells showed a high invasive capacity in the presence of matrigel. They were incubated in serum-free medium with low-insulin concentrations and without chemoattractant at the lower chamber of the insert. LNCaP cells were incubated in medium with low-serum concentrations (2–3%) using 10% FCS as chemoattractant. After 48 h, the cells, which invaded through the matrigel layer, were stained with DAPI and counted using a fluorescent microscope (Zeiss, Axiovert, Jena, Germany) and TissueFAXS software package (TissueGnostics, Vienna, Austria).

Analysis of IGFBP-3 membrane binding by flow cytometry

Prostate cancer cells were suspended in PBS, cooled to 4 °C for 1 h to reduce endocytosis, and incubated with human recombinant IGFBP-3 for 30 min. Unbound protein was removed by washing the cells with PBS following fixation in 3.5% paraformaldehyde. Cellular permeabilization using PBS supplemented with 0.5% Tween 20 was performed for control experiments.

Immunofluorescent staining of membrane-bound IGFBP-3 was performed by using either a rabbit polyclonal (Upstate Biotechnology, Lake Placid, NY, USA) or a mouse monoclonal antibody (R&D Systems). The intracellular antigen cytokeratin was stained using a mouse monoclonal antibody (Chemicon Europe, Hampshire, UK) and served as a control. Negative controls were performed with either mouse or rabbit isotype control antibodies (Dako, Glostrup, Denmark). Primary antibodies were diluted 1:500 in PBS and incubated for 1 h. Secondary ALEXA 555-labeled antibodies (Invitrogen) were diluted 1:500 in PBS and incubated for 30 min. Each incubation step was followed by a washing step with PBS. Finally, the cells were analyzed using FACSCalibur (Becton, Dickinson, San Jose, CA, USA).

Western-blot analysis

For Western-blot analysis, we used cellular lysates from apoptosis assays (see cell apoptosis assay). Alternatively, cells were plated in six-well plates at a density of 500 000 cells per well. When the cells reached 70% of confluence, they were starved for 24 h and stimulated with 25 ng/ml of IGF-I for 15 min in the presence or absence of IGFBP-3. Cells were harvested and lysed in glycine-SDS sample buffer (Gradipore, Frenchs Forest, NSW, USA). Total protein was quantified using the Bradford method. About 50 µg of protein per lane were then resolved using a 4–12% Bis-Tris gel (Invitrogen) and transferred onto a nitrocellulose membrane (Invitrogen). The membrane was blocked for 1 h using Starting Block (tris-buffered saline, TBS) buffer (Pierce Biotechnology, Rockford, IL, USA) and incubated at 4 °C overnight with the antibodies pAKT (phosphorylated oncogene AKT-1, rabbit, Cell Signaling, Heidelberg, Germany; dilution 1:500) and AKT (goat, Santa Cruz Biotechnology, Heidelberg, Germany; dilution 1:500) or pERK1/2 (phosphorylated extracellular signal regulated kinases 1 and 2, mouse, Cell Signaling; dilution 1:300), ERK1 (rabbit, Santa Cruz; dilution 1:300) and ERK2 (rabbit, Santa Cruz Biotechnology; dilution 1:300), or cleaved PARP (PARP p85 fragment, rabbit, Promega; dilution 1:1000) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, mouse, Chemicon; dilution 1:10 000). This step was followed by incubation with fluorescence-labeled secondary antibodies (Molecular Probes, Invitrogen) for 1 h at room temperature. The membranes were scanned using the Odyssey Infrared Imaging System and densitometric analysis was performed by using Odyssey application software (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Statistics were performed by using SPSS 12.0 (SPSS, Chicago, IL, USA) and Microsoft Excel 2002 for Windows. Gaussian distribution of all data sets was confirmed using Kolmogorov–Smirnov test. Differences among treatment groups were analyzed using two-sided Student's *t*-test. *P* values below 0.05 were considered significant. In addition, statistics were controlled using Mann–Whitney *U* test, a nonparametric test identifying statistically significant differences independent of sample distribution and variance between the samples. All differences highlighted by asterisks were statistically significant also with the Mann–Whitney *U* test. Data are presented as mean $\pm$ s.d. unless otherwise stated, *P* values are encoded in the figures as **P* < 0.05; ***P* < 0.01.

Results

IGFBP-3 inhibits IGF-induced cell proliferation and exerts long-term IGF-independent inhibitory effects on prostate cancer cells

IGFs are well-known growth factors for prostate cancer cells. The binding of IGFs to their receptors leads to an increased proliferation and survival signal in tumor cells, which is mediated via the activation of different intracellular-signaling pathways, including the phosphatidylinositol-3-kinase (PI3K)/AKT pathway as well as the mitogen-activated protein kinases (MAPK)/ERK pathway. The activity of AKT is controlled by the

tumor suppressor phosphatase and tensin homolog (PTEN). PTEN is mutated or lost in many prostate cancer cells.

To monitor the ability of IGFBP-3 to inhibit IGF-I-induced activation of signaling pathways in prostate cancer cells, we analyzed the activation of the PI3K/AKT pathway and the MAPK/ERK pathway upon IGF-I treatment in the presence or absence of IGFBP-3 using immunological detection of phosphorylated AKT and ERK (Fig. 1). In PTEN-positive Du145 cells, AKT and ERK phosphorylation was induced upon IGF-I stimulation and induction was abrogated by the addition of IGFBP-3. The two PTEN-negative cell lines PC3 and LNCaP showed a high basal AKT phosphorylation that could not be further stimulated by IGF-I. This result is in agreement with the study of Lackey *et al.* (2007) who showed that loss of PTEN by experimental knockdown caused loss of sensitivity to IGF-I signaling. Likewise, the phosphorylation of ERK was not induced upon IGF stimulation in PTEN-negative cells and the addition of IGFBP-3 had no effect on that pathway.

Next, we examined the ability of IGFBP-3 to inhibit the growth-stimulatory signal of IGF. We treated prostate cancer cells with IGF-I for 3 days and measured cellular proliferation by determination of total cell numbers (Fig. 2 and the Electronic Supplementary Fig. 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). IGF-I stimulated the cell proliferation of Du145 prostate cancer cells. In the presence of IGFBP-3, this IGF-mediated growth stimulation was inhibited.

Figure 1 IGFBP-3 blocks IGF-mediated intracellular signaling. IGFBP-3 inhibits IGF-induced AKT and ERK phosphorylation in PTEN-positive cells. Cells were serum starved for 24 h and stimulated with 25 ng/ml IGF-I for 15 min in the presence or absence of IGFBP-3. The phosphorylation of AKT and ERK proteins was detected by Western-blot analysis. The activation by IGF and inhibition of activation by IGFBP-3 were seen only in Du145 cells possessing a functional PTEN. PC3 and LNCaP cells possessing mutant PTEN showed a constitutively activated AKT pathway and were unresponsive to IGF. (A) Representative Western blot. (B) Western-blot quantification by densitometry, *n* = 3, bars represent mean $\pm$ s.d., and significant differences are highlighted with **, when *P* < 0.01.

Figure 2 IGFBP-3 inhibits cell proliferation. IGFBP-3 blocks IGF-mediated cell proliferation and inhibits cell proliferation in an IGF-independent manner after long-term incubation and one cellular passage. (A) Proliferation of prostate cancer cells cultured in the presence or absence of IGF and IGFBP-3 was analyzed by determination of total cell number after 3 and 7 days of incubation. After 4 days of culture (3 days of treatment), the cells reached confluence and were reseeded at equal cell numbers in new culture wells. Controls performed with IGFBP-5 and insulin, an IGF-related growth factor, are shown in B. Graphs represent mean $\pm$ s.d. of four independent experiments performed in at least three parallels, and significant differences are highlighted with **, when $P < 0.01$.

PC3 and LNCaP (PTEN negative) were not growth stimulated by IGF-I and the addition of IGFBP-3 had no effect on their cell proliferation. To determine long-term effects of IGFBP-3 on cell proliferation, we treated prostate cancer cells over 7 days with IGFBP-3. The cells reached confluence after 4 days and were reseeded at equal cell numbers in new culture plates. After a prolonged treatment of 7 days and one cellular passage, we observed an inhibitory effect of IGFBP-3 on cell proliferation in all the tested cell lines at all conditions (Fig. 2). This inhibitory effect was specific for IGFBP-3 because recombinant IGFBP-5 had no effect on cell proliferation. Together, these data indicate neutralization of IGF effects on proliferation by IGFBP-3 and in addition IGF-independent long-term growth inhibitory effects of IGFBP-3 on prostate cancer cells including IGF-insensitive cells. Inhibition of endogenous IGF cannot be involved in the observed long-term inhibitory effects since the prostate cancer cell lines, except LNCaP cells, produce only minute amounts of IGFs (see Supplementary Fig. 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>).

IGFBP-3 was reported to induce or enhance apoptosis in cellular cancer models (reviewed in Butt & Williams (2001)), and therefore we investigated whether the anti-proliferative effects of IGFBP-3 may be mediated by induction of apoptosis. We exposed prostate cancer cells to IGFBP-3 and IGF-I for 72 h and analyzed apoptosis by measuring activated

caspase-3 activity and detection of cleaved PARP by immunoblot. Under our experimental conditions, no apoptosis was induced upon IGFBP-3 treatment in Du145 (Fig. 3) and the two other cell lines (data not shown). Next, we examined whether IGFBP-3 increases the apoptotic response of cells treated with cytotoxic agents. We treated prostate cancer cells with etoposide (inhibitor of topoisomerase II) or cycloheximide (inhibitor of protein synthesis) for 48 h in the presence and absence of IGFBP-3. Whereas the cytotoxic agents generated a significant induction of apoptosis, the presence of IGFBP-3 had no additive effect in Du145 (Fig. 3) and the two other cell lines (data not shown). Thus, exogenously applied recombinant human IGFBP-3 did not induce apoptosis in prostate cancer cells and did not potentiate the effects of cytotoxic substances in our experimental system.

IGFBP-3 inhibits cell-matrix adhesion

Since we were interested on processes involved in prostate cancer progression and metastasis formation, we investigated the influence of IGFBP-3 on cell adhesion. We seeded cells in the presence and absence of IGFBP-3 and determined the number of adherent cells after 2 and 6 h (Fig. 4). IGFBP-3 decreased the cell adhesion in all tested cell lines under all conditions. The inhibitory effect of IGFBP-3 on cell attachment was significant 2 h after seeding in all cell lines and in Du145 and LNCaP cells still detectable 6 h

Figure 3 IGFBP-3 has no effect on prostate cancer cell apoptosis. (A) Du145 cells were treated with IGF-I and IGFBP-3 for 72 h. Apoptosis was measured using caspase-3 assay kit (left diagram) and anti-cleaved PARP immunoblot (right graph). (B) Du145 cells were exposed to different apoptotic stimuli for 48 h and apoptosis was determined in the presence or absence of IGFBP-3. Cellular apoptosis increased significantly in the presence of apoptotic agents. The presence of IGFBP-3 had no additional effect. Bars represent mean $\pm$ S.D. of a minimum of three independent experiments, and significant differences are highlighted with **, when $P < 0.01$.

after seeding (Fig. 4A). At this time point, PC3 cells had already completely adhered to the culture surface (Fig. 4A and C). The inhibitory effect of IGFBP-3 on cell adhesion was similar in the presence and absence of IGF-I. Controls were performed with HBP, IGFBP-5, and MAB391, an antibody blocking the IGF-I receptor. None of these treatments had any effect on cell attachment indicating a specific, IGF-independent effect for IGFBP-3 (Fig. 4B).

To further characterize this IGFBP-3 effect, we performed additional experiments to reveal whether adhesion to extracellular matrix components and cell–cell adhesion were impaired in the presence of IGFBP-3. We first seeded fluorescently labeled Du145 cells on 96-well culture plates that were coated with the different extracellular matrix components gelatin, laminin, fibronectin, and polylysine. After 2 h and two washing steps, adherent cells were measured using a fluorescent microplate reader. As shown in Fig. 4D, IGFBP-3 reduced the adhesion of prostate tumor cells to all extracellular matrix components tested.

Next, we analyzed whether IGFBP-3 has any influence on the adhesion of tumor cells to endothelial cells, which represents a crucial step in the process of metastasis formation. In epithelial/endothelial interaction assays, we tested the ability of prostate cancer cells to bind to HBMEC. PC3 cells showed the highest adhesion capacity to HBMEC, followed by Du145 and LNCaP. The presence of IGFBP-3, however, had no influence on epithelial/endothelial

cell adhesion (Fig. 4E). In summary, our data show that IGFBP-3 has a clear inhibitory effect on cell–matrix adhesion but does not influence cell–cell adhesion.

Based on the results of these experiments, we wondered whether the influence of IGFBP-3 on cell–matrix adhesion is mediated by an extracellular action of the protein. IGFBP-3 was shown to exert extracellular functions as well as functions that are mediated by internalization of the protein via distinct endocytic pathways, which can be blocked by chemical inhibitors of endocytosis (Lee *et al.* 2004). To evaluate whether endocytosis is required for the inhibitory actions of IGFBP-3 on cell adhesion, we preincubated prostate cancer cells with chemical compounds that inhibit clathrin-mediated (chlorpromazine and amantadine) or caveolae-mediated (nystatin, cyclodextrin, and genistein) endocytosis (Van Hamme *et al.* 2008) or combinations inhibiting both pathways. None of these inhibitors, alone or in combination, affected reduction in cellular attachment by IGFBP-3, suggesting that internalization of IGFBP-3 is not required for its inhibitory effects on cell adhesion (Fig. 4F).

In order to analyze whether IGFBP-3 binds to the surface of prostate cancer cells, we incubated cells with recombinant IGFBP-3, fixed the cells, and analyzed cell surface binding of the protein using flow cytometry analysis (Fig. 5). To control for specific staining of proteins on the cell surface, we also stained in parallel for cytokeratin, an intracellular cytoplasmic protein, which was not detected under the conditions of our

Figure 4 IGFBP-3 inhibits prostate cancer cell-matrix adhesion. (A–C) IGFBP-3 inhibits the attachment of prostate cells. (A) Cells were seeded in six-well culture dishes. After 2 h and 6 h, the floating cells were removed by washing twice with PBS and adherent cells were counted. IGFBP-3 inhibits cell attachment in the presence and absence of IGF. (B) Controls performed with heparin-binding protein (HBP), IGFBP-5, and MAB391 (blocking antibody for IGF-I R) did not have any effect on cell adhesion. (C) Percentage of adherent cells. Out of the three cell lines tested, PC3 adhered fast with more than 80% attached cells after 6 h, followed by Du145 and LNCaP. (D) IGFBP-3 inhibits cell adhesion to extracellular matrix components. Cells were labeled with the fluorescent dye calcein AM and seeded in 96-well plates coated with different molecules as indicated. Nonadherent cells were removed after 2 h by washing with PBS and adherent cells were measured using a fluorescence plate reader. (E) IGFBP-3 has no influence on epithelial–endothelial cell adhesion. Calcein AM-labeled cancer cells were seeded on a confluent monolayer of human bone marrow endothelial cells (HBMEC). Nonadherent cells were removed by washing after defined time points as indicated and adherent cells were measured. (F) Inhibitors of endocytosis do not reverse the inhibitory effect of IGFBP-3 on cell adhesion. Cells were treated with chemical inhibitors of clathrin-mediated endocytosis pathway (chlorpromazine and amantadine) and caveolae-mediated endocytosis pathway (nystatin, β -cyclodextrin, and genistein) or combinations inhibiting both pathways for 45 min. Afterwards, they were seeded in the presence and absence of IGFBP-3, and cellular adhesion after 2 h was analyzed. None of the used endocytosis inhibitors was able to block the inhibitory effect of IGFBP-3, showing that internalization of IGFBP-3 is not required for its effects on cell adhesion. Data of all graphs represent mean $\pm$ s.d. of at least three independent experiments in (A–D and F) and two independent experiments in (E); significant differences are highlighted with **, when $P < 0.01$.

assay (Fig. 5B). The results clearly show that exogenously added IGFBP-3 binds to the cell surface of prostate cancer cells (Fig. 5A).

IGFBP-3 inhibits cancer cell motility

To investigate whether IGFBP-3 influences not only adhesion, but also motility of metastatic prostate cancer cells, we determined the movement of prostate cancer cells using a live-cell imaging technique. The movement of prostate cancer cells was followed over a period of 12 h, and the cumulative distance of cellular

migration performed by the cells was measured by manual cell tracking of time-lapse recordings (Fig. 6). Out of the three prostate cancer cell lines tested (Du145, LNCaP, and PC3), only PC3 showed sufficient movement to be analyzed in this assay. Since we monitored motility by time-lapse recordings of routine cultures, motility was non-directed. The optimal time point to investigate PC3 motility was found to be 12 h after seeding, when all cells, including those treated with IGFBP-3, had already adhered to the culture surface and had formed their characteristic shapes. In the presence of IGFBP-3, the

Figure 5 IGFBP-3 binds to the cell surface of prostate cancer cells. (A) Prostate cancer cells were incubated with recombinant IGFBP-3 at 4 °C for 30 min. Unbound protein was removed by washing the cells with PBS prior to fixation with paraformaldehyde. Staining was performed using an anti-IGFBP-3 primary monoclonal antibody and ALEXA 555-labeled secondary antibody and analyzed by fluorescent flow cytometry (FACS). Cellular permeabilization was avoided in this experiment to ensure specific cell surface staining. The prostate tumor cells showed some basal IGFBP-3 staining (compared with the isotype controls), and incubation with recombinant IGFBP-3 increased the surface staining signal. A different polyclonal primary antibodies showed a similar staining pattern (data not shown). (B) The experimental conditions and the specificity for surface staining were controlled by staining of a cytoplasmic antigen (cytokeratin) in unpermealized cells (treated as cells in A) and permealized cells. Unpermealized cells showed no cytokeratin staining confirming that the experimental conditions in (A) are adequate to analyze cell surface binding of IGFBP-3. Data represent mean $\pm$ S.D. of three independent experiments in (A) and two independent experiments in B.

movement of PC3 cells was 1.7-fold slower than the movement of control cells, and the total distance of migration was only 60% of that of control cells ($P < 0.001$). This effect of IGFBP-3 was also found in the presence of IGF-I and epidermal growth factor (EGF; Fig. 6C). While IGF-I had no effect on cell motility, EGF, used as positive control, was able to increase the velocity of PC3 cells. Controls performed with HBP and MAB391 (IGF-I receptor-blocking antibody) had no effect on cell movement. IGFBP-5 decreased the PC3 motility; however, the effect was smaller compared with the IGFBP-3 effect. These data show that IGFBP-3 inhibits the motility of fast-moving prostate cancer cells.

IGFBP-3 inhibits cancer cell invasion through matrigel

Next, we examined whether IGFBP-3 influences prostate cancer cell invasion. Cancer cells were plated on matrigel invasion chambers and incubated for 48 h. Cell invasion was analyzed by the detection of cells that invaded through the matrigel layer to the lower surface of the invasion chambers (Fig. 7). IGFBP-3 inhibited invasion of Du145 cells (Fig. 7A and B). This inhibitory effect of IGFBP-3 was also detected in the presence of IGF-I. Controls performed with HBP, IGFBP-5, and MAB391 had no effect on Du145 invasion through matrigel. PC3 and LNCaP showed no significant difference in their invasion properties in the presence of IGFBP-3 (Fig. 7C). These data show that IGFBP-3 can inhibit prostate tumor cell invasion in some cellular models of metastatic prostate cancer.

Discussion

IGFBP-3 is a multifunctional protein that was described to inhibit growth and enhance apoptosis in an IGF-dependent as well as in an IGF-independent way in several tumor models. We could confirm the inhibitory action of IGFBP-3 on IGF-mediated cell proliferation. This effect is possibly a result of an inhibition of IGF binding to its cell surface receptors by the formation of IGF/IGFBP complexes as previously described (Jones & Clemmons 1995, Hwa *et al.* 1999). In addition, we also observed an IGF-independent effect of IGFBP-3 on tumor cell proliferation after long-term incubation and one cellular passage. In comparison to this, the short-term anti-proliferative effect of IGFBP-3 was dependent on IGF-I and therefore seen only in the IGF-I-sensitive Du145 cells. By contrast, the long-term growth-inhibitory effect of IGFBP-3 was seen in all three cell lines, notably also in those that have lost PTEN function and have a constitutively activated PI3K/PKB/AKT pathway. This is of importance when therapeutic use of IGFBP-3 is considered since PTEN mutations are quite common in prostate tumors (Sansal & Sellers 2004).

Under our experimental conditions, we did not observe an effect of IGFBP-3 on prostate cancer cell viability. These findings contrast studies that showed IGFBP-3-mediated apoptosis (Hong *et al.* 2002, Bhattacharyya *et al.* 2006, Zappala *et al.* 2008). Several of the reported studies involved IGFBP-3 overexpression, and in some cases mutated versions of the protein had been used. Apparently, these experimental settings do not really reflect the *in vivo*

Figure 6 IGFBP-3 inhibits prostate cancer cell motility. PC3 cells were incubated under a Cell-IQ live-cell imaging platform, and cellular movement was followed and analyzed using ImageJ software and the special plug-in Manual Tracking. (A) To illustrate how the analysis was performed, the movement of a PC3 cell in 12 h is shown (a, position of the cells at the start point of the analysis; b, position of the cell 12 h later; c, moved distance shown with a white line, white squares represent the respective positions of the cell after every 10 min). (B) Movement of PC3 cells in the course of time. The cellular movement was slower in the presence of IGFBP-3. (C) Mean distance covered by PC3 cells in 12 h. Pictures were taken using a tenfold objective and an imaging interval of 10 min. Data represent mean of 20 representative cells tracked in two different experiments. Error bars show standard error in (B) and s.d. in (C), and significant differences are highlighted with **, when $P < 0.01$.

situation, where IGFBP-3 is expressed, produced, and secreted by all prostate cells with a major contribution by stromal cells, as we have previously shown (Massoner *et al.* 2008). This suggests mainly an exogenous exposure of epithelial and tumor cells to IGFBP-3 *in vivo*. We believe that the observed long-term inhibition of proliferation is significant for the *in vivo* situation with continued exposure to the protein, representing ideal conditions for possible therapeutic applications.

Apart from its role as negative regulator of proliferation, we explored an additional very important and so far poorly described function of IGFBP-3, where IGFBP-3 can negatively influence adhesion, motility, and invasion processes of prostate tumor cells. This, we expect to have implications for invasive and metastatic properties of prostate tumors.

IGFBP-3 is expressed, produced, and secreted by all prostate cells with a major contribution by stromal cells (Massoner *et al.* 2008). Once secreted, the protein can be found in the extracellular matrix, where IGFBP-3 was shown to bind to different extracellular matrix components like collagen (Liu *et al.* 2003), fibronectin (Martin & Buckwalter 2000, Gui & Murphy 2001), and proteoglycans (Baxter 1990, Smith *et al.* 1994). Extracellular IGFBP-3 was shown to bind to the cell surface (Oh *et al.* 1993, Smith *et al.* 1994) and we confirmed this finding. Surface binding of IGFBP-3 was described as occurring via cell membrane-associated glycosaminoglycans, especially heparin

and heparan sulfate (Baxter 1990, Yang *et al.* 1996, Beattie *et al.* 2005), or via specific molecules like integrin- $\beta 1$ (Burrows *et al.* 2006) and (as a complex with transferrin) the transferrin receptor (Lee *et al.* 2004). The long-standing search for IGFBP-cell surface receptors has not yet provided definitive results and a specific receptor for IGFBP-3 has not been confirmed so far. Many actions of IGFBP-3 are mediated by internalization of the protein via distinct endocytic pathways including caveolae- and clathrin-mediated (specifically transferrin/transferrin receptor 1) endocytosis. Inhibition of these pathways was shown to specifically inhibit IGFBP-3 uptake (Lee *et al.* 2004). For the inhibitory functions of IGFBP-3 on cell adhesion described here, IGFBP-3 uptake was not required since inhibition of endocytosis did not influence its effects. A similar influence of IGFBP-3 on cell adhesion has previously been described also in some cellular models of breast cancer (McCaig *et al.* 2002), thus supporting our data. We speculate that its binding to the cell surface influences molecules involved in the complex process of cellular adhesion via direct or indirect binding or masking of molecules in the extracellular matrix. However, the distinct mechanisms of how IGFBP-3 interferes with cell adhesion need to be further analyzed.

IGFBP-3 does not only influence adhesion, but also the motility of prostate cancer cells. The process of cellular movement of tumor cells has not been fully elucidated yet, but it is considered to occur via

Figure 7 IGFBP-3 inhibits Du145 invasion through matrigel. Cells were seeded in chambers containing an 8 μ M pore matrigel-coated membrane. The invasive capacity of cancer cells was investigated by determining the number of cells that were able to invade through the matrigel layer. (A) IGFBP-3 can inhibit Du145 invasion through matrigel in the presence and absence of IGF. (B) Controls performed with HBP, IGFBP-5, and MAB391 did not have any effect on Du145 cell invasion, showing that the effect is specific for IGFBP-3. (C) IGFBP-3 has no effect on PC3 and LNCaP invasion. Data represent mean $\pm$ s.d. of at least five independent experiments, and significant differences are highlighted with **, when $P < 0.01$.

cellular pseudopodia formed by the actin cytoskeleton that enables the cells to migrate along extracellular matrix components. Cell motility is considered to be a continuum of sequential events in which the cell extends pseudopodia, forms nascent attachments, assembles and contracts the cytoskeleton, and finally, as it translocates forward, disengages distal adhesions (Matsumoto *et al.* 1995). Several molecules that were described to bind IGFBP-3 are also involved in motility processes, like proteoglycans (Cattaruzza & Perris 2005) and integrins (Matsumoto *et al.* 1995,

Juliano *et al.* 2004, Ramsay *et al.* 2007). At our present stage of knowledge, it remains unclear whether the inhibitory actions of IGFBP-3 on cell adhesion and on cell motility are due to the same molecular interactions or reflect two distinct processes. In concordance with our data, exogenous IGFBP-3 was also described to significantly reduce the migration of Ewing's sarcoma cell lines (Benini *et al.* 2006) and nonsmall cell lung cancer cell lines (Oh *et al.* 2006).

Furthermore, we found that IGFBP-3 inhibits tumor cell invasion. However, this effect was not seen in all cell lines tested. Many proteases in the prostatic tissue were described to degrade IGFBP-3, including prostate specific antigen (PSA) (Cohen *et al.* 1992). LNCaP cells produce and secrete PSA. Therefore, we analyzed whether extracellular IGFBP-3 was degraded by LNCaP. But in none of our *in vitro* cell models, we observed a substantial degradation of extracellular IGFBP-3 (data not shown), indicating that the differences seen between Du145 cells on one hand and PC3 and LNCaP cells on the other hand are not due to differences in processing of IGFBP-3. IGFBP-3 acts as an invasion-suppressor molecule for Du145 cells, and it needs to be further investigated whether IGFBP-3 is also able to modulate *in vivo* invasion of tumor cells as it has been recently shown for ovarian endometrioid cancer (Torng *et al.* 2008).

In summary, we could show in this work that IGFBP-3 is not only involved in growth processes of the prostate and prostate cancer, but it can also influence adhesion, motility, and invasion of prostate cancer cells. This is a newly described mechanism by which IGFBP-3 might influence tumor progression to an advanced disease. These findings may be of considerable importance in the process of metastasis formation and support the use of IGFBP-3 in therapeutic settings.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work and the work was not supported by any company or industrial organization.

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Author contribution statement

Petra Massoner: performance of all *in vitro* assays including proliferation, apoptosis, adhesion, motility and invasion assays (in parts), Western blot, real-time PCR, and flow

cytometry analysis. Project concept and planning (in parts), data analysis, statistics, and manuscript preparation (figures and text). *Daniela Colleselli*: performance of a part of the invasion assays. *Andrea Matscheski*: purification of human recombinant IGFBP-3 (establishment of the technique). *Haymo Pircher*: purification of human recombinant IGFBP-3. *Stephan Geley*: assistance with life cell imaging and motility assays, constructive discussions. *Pidder Jansen Duerr*: supervision of purification of human recombinant IGFBP-3 and constructive discussions. *Helmut Klocker*: general idea and project concept, supervision of all experiments, methodological assistance and interpretations of the results, manuscript revision, and funding.

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