Abstract. Urokinase-type plasminogen activator (uPA) and its specific membrane receptor (uPAR) control extracellular matrix proteolysis, cell migration, invasion and cell growth in several cancers. The uPAR released from human cancers is detected in blood as soluble uPAR (suPAR). No information is available on the mechanism(s) of action of suPAR on prostate cancer (PCa) cell growth and invasion. In order to clarify this issue, we tested the effect of a treatment with the human recombinant suPAR (comprising amino acids 1-303) on the proliferation, migration and invasion of DU145 cells, a PCa cell line expressing a potent autocrine uPA-uPAR signalling system. The results indicate that suPAR significantly inhibits cell growth, promotes apoptosis and decreases both migration and Matrigel™ invasion of DU145 cells. The mechanism of action of suPAR seems to be linked to a decrease of ERK and FAK activation. Cleavage of suPAR by chymotripsin reverses these effects. When added to the uPA-negative LNCaP cells, suPAR was ineffective; on the contrary, when LNCaP cells were cultured on fibronectin-coated plates in order to stimulate uPA expression, suPAR significantly decreased cell proliferation. In conclusion, our data suggest that suPAR can function as a potent molecule scavenger for uPA in human PCa cells characterized by high levels of uPA/uPAR as in DU145 cells, while it is ineffective in uPA-deficient LNCaP cells. The molecular mechanism(s) through which suPAR participates in the control of PCa progression may bear relevance for the long-term goal to identify new therapeutic targets aimed at silencing tumours in vivo.

Introduction

Advanced prostate cancer (PCa) morbidity and mortality are strictly correlated with a higher proliferation rate and metastatic progression (1). Little information is available on the molecular mechanism(s) involved in the control of the metastatic properties of PCa cells.

The processes of tissue remodelling associated with cancer invasion and metastasis are controlled by proteolytic enzymes, which participate in the extensive disruption of cell/cell and cell/extra-cellular matrix (ECM) contacts (2). The tumour cell-associated urokinase-type plasminogen activator (PA) system, consisting of the serine protease uPA, its substrate plasminogen, the membrane-bound receptor uPAR, as well as the inhibitors PAI-1/2, plays an important role in these pericellular processes and it is implicated in PCa progression and invasion (3-5).

uPA is secreted as an inactive pro-enzyme, which localizes on the cell surface by binding uPAR (6). The human receptor uPAR consists of 3 homologous domains (D1, D2 and D3) linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor at the C-terminus (7). Since the amino-terminal domain (D1) is required for the binding to uPA, all three domains are needed for high affinity binding (8,9).

The presence of the uPA/uPAR complex provides by the tumour cells may be sufficient to modulate directional proteolysis through the activation of plasminogen, initiating a proteinase cascade which culminates in metalloproteinase activation and degradation of extracellular matrix proteins (10-13).

uPAR laterally associates with several trans-membrane receptors, including integrins and the EGFR within the same plasma membrane. By regulating the activity of integrins and EGFR, uPAR also participates in the control of extracellular signals controlling cell growth (14).

Overexpression of components of the uPA-system in non-metastatic cells enhances metastasis; inhibition of uPA and/or of the uPA/uPAR interaction prevents or reduces metastasis in animal models (22,23). Reduced metastasis of transgenic
mammary cancer is reported in urokinase deficient mice (24). Furthermore, antimitastatic drugs such as GnRH analogues widely used for PCa treatment exhibit not only an anti-proliferative effect, but also inhibitory effects on uPA secretion (25).

A soluble form of uPAR (suPAR) lacking the GPI-anchor has also been found both in vivo and in vitro. Intact uPAR is released from the plasma membrane by GPI-specific phospholipase and proteases (26) and may also be generated by alternative splicing of the uPAR mRNA (27). Cleaved uPAR forms are present in several neoplastic cell lines and tissues (28,29). In humans, suPAR, both intact and cleaved, is found in blood, ascitic fluid, tissues and urine of many types of cancer. Measurements of suPAR forms in squamous cell lung cancer (30), colon cancer (31) and breast cancer (32) have significant diagnostic importance, since increased uPAR in blood is correlated with poor prognosis (33,34). However, uPAR has not been shown to be useful in PCa diagnostics (13,35), even if measurements of individual cleaved forms of suPAR contributed significantly to discrimination of men with PCa from those with no evidence of malignancy (36,37). In animal models, suPAR reduces in vivo the growth and metastasis of MDA-MB 231 breast cancer cells and OV-MZ-688 ovarian cancer cells (38,39).

No information has been published so far regarding a possible role of intact suPAR in the mechanisms controlling PCa progression.

To this purpose, we have studied the effect of suPAR on cell growth, migration and invasion of PCa cells; the signalling pathways affected by suPAR, especially in terms of ERK and FAK activation, have been tested. Experiments have been performed on PCa metastatic and androgen-independent DU145, expressing high levels of uPA/uPAR. Moreover, we have tested the effect of suPAR on cell growth of androgen-dependent LNCaP; in these cells, uPAR is not expressed at a sufficient level to establish autocrine uPA/uPAR signalling.

Materials and methods

Reagents and antibodies. Recombinant human soluble uPAR (suPAR), comprising the amino acids 1-303, was from R&D Systems, Inc. (Minneapolis, MN). suPAR was dissolved in sterile PBS containing 0.1% bovine serum albumin to prepare a stock solution of 20 μg/ml.

Cleaved suPAR (CsuPAR) was prepared by treating suPAR with 2.0 nM chymotrypsin for 7 h at 37°C. The chymotrypsin was inactivated with 1.0 mM PMSF, as described (40). Chymotrypsin and protease inhibitor mixture were from Sigma-Aldrich (Milano, Italy). Antibodies to ERK1/2, FAK, uPA and uPAR were from Santa Cruz (CA). Horseradish peroxidase conjugated antibodies were from Santa Cruz (CA).

Cell culture. The PCa DU145 and LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were routinely grown in RPMI-1640 medium (Biochrom KG, Berlin, Germany), supplemented with 5% foetal bovine serum (FBS) that was obtained from Gibco BRL, Grand Island, NY, glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂, 95% air at 37°C.

In order to stimulate uPA production, the LNCaP cells were plated in tissue culture wells pre-coated with fibronectin (5 μg/ml, BD Biosciences).

Cell growth studies. DU145 cells, plated in 10-mm dishes in serum-free medium, were treated for 48 h with suPAR (1.0-10 nM). Cells were then harvested and counted by using a haemocytometer. In another series of experiments, 1 μCi/ml [³H]thymidine was added to DU145 and LNCaP cells. DU145 cells, cultured in serum-free medium, were incubated for 24 h with graded doses of suPAR (1.0-10 nM). Six hours following [³H]thymidine addition, cells were washed in PBS and radioactivity was counted in a β-counter 1600CA TRI-CARB (Perkin-Elmer).

The results of three separate experiments are presented as the mean ± SD. Each experimental group was composed of 8 replicates. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison tests. p<0.05 was considered statistically significant.

FACScan analysis. For FACScan analysis, adherent DU145 cells, pre-treated with suPAR (10 nM) for 24 h in serum-free medium, were harvested, pooled with the culture supernatant containing the apoptotic cells already detached from the dish and centrifuged. Cells (1x10⁶) were washed in PBS, fixed in 70% ethanol, centrifuged, and re-suspended in DNA staining solution (PBS containing 200 mg/ml RNase A, 20 mg/ml propidium iodide plus 0.1% Triton X-100). Cells were then stained by incubation at room temperature for 60 min. All cells were then measured on a FACScan flow cytometer (Becton Dickinson, UK) with an argon laser at 488 nm for excitation and analyzed using Cell Quest software (Becton Dickinson). All the flow cytometric measurements were made using the same instrument settings, and at least 10,000 cells were measured in each sample. A quantifiable peak detected apoptotic cells in the sub-G1 phase corresponding to the red fluorescence light emitted by sub-diploid nuclei of cells; the results were expressed as the percentage of death by apoptosis induced by suPAR treatment. The results of three separate experiments are presented as the mean ± SD. Each experiment was performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison tests; p<0.05 was considered statistically significant.

Migration and invasion assays. Briefly, cell migration assay was performed using a 48-well Boyden chamber (Neuroprobe, Inc) containing 8-μm polycarbonate filters (Nucleopore, Concorezzo, Milan, Italy). Filters were coated on one side with 50 μg/ml laminin, rinsed once with PBS, and then placed in contact with the lower chamber containing RPMI-1640 medium. DU145 cells, pre-treated for 48 h with suPAR (1.0-10 nM), were collected and then added in aliquots (75,000 cells/50 μl) to the top of each chamber and allowed to migrate through coated filters for 4 h. At the end of the incubation, the migrated cells attached to the lower membrane surfaces were fixed, stained with DiffQuik (Biomap, Italy) and counted at a x40 magnification by standard optical microscopy.

Invasion assay was performed in invasion chambers (Becton Dickinson, Bedford, MA) containing a membrane coated with Matrigel™. The digestion of Matrigel allowed...
the migration of cancer cells. Briefly, DU145 cells, pre-treated with suPAR (1.0-10 nM) for 48 h, were collected and 500 μl of cell suspension (75,000 cells) was added to trans-well inserts with an 8-μm pore size coated with Matrigel. In the lower compartment of the invasion chamber 5% FBS-containing medium was added as a chemo-attractant. After a 22-h incubation period at 37˚C, cells that passed through the Matrigel-coated filter into the bottom wells were fixed, stained with Diffquik (Biomap, Italy) and counted at a x40 magnification by standard optical microscopy.

The results of three separate experiments are presented as the mean ± SD. Each experimental group was composed of 8 replicates. Cell cycle analysis of DU145 cells treated with suPAR (C and D). The adherent DU145 cells were treated for 24 h with suPAR (10 nM) in serum-free medium. All cells were then measured on a FACScan flow cytometer with an argon laser at 488 nm for excitation and analyzed using Cell Quest software. The results were expressed as the percentage of cell death by apoptosis induced by suPAR treatment. Each experiment was performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison tests. *p<0.05 vs controls was considered statistically significant.

Immunoblot analyses. DU145 cells plated in 10-mm dishes in serum-free medium were treated with human recombinant suPAR (10 nM) or CsuPAR (10 nM), as indicated. DU145 cells were harvested in RIPA buffer containing AEBSF (0.4 μg/μl), leupeptin (1 μg/μl) and pepstatin A (1 μg/μl), centrifuged, and washed in PBS. Protein concentration was determined using the Bradford assay. Equal amounts of protein (50 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), for 1.5 h at 110 V. Proteins were blotted using transfer apparatus (BIO-RAD Trans Blot semi-dry). The membrane was washed with 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 (TBST) for 30 min, immersed in a blocking solution with TBST and 5% (w/v) dry skimmed milk, and then incubated with a diluted solution of the primary antibody at 4˚C over night.

LNCaP cells were plated in 24-well plates pre-coated or not with fibronectin (5 μg/ml) for 48 h. Cultures were then washed with serum-free medium and incubated for 24 h with suPAR (10 nM). The cell supernatants, after gel electrophoresis (SDS-PAGE), were subjected to immunoblot analysis to detect uPA.

For ERK1/2, FAK and uPA analyses, we used 1:100 mouse monoclonal antibodies against P-ERK1/2 (E-4, Santa Cruz), 1:1000 rabbit polyclonal antibody against ERK1/2 (K-23, Santa Cruz), 1:100 rabbit polyclonal antibodies against P-FAK (Ser 722, Santa Cruz), 1:100 rabbit polyclonal antibodies against FAK (A-17, Santa Cruz) and 1:100 goat polyclonal antibody against uPA (C-20, Santa Cruz).

After incubation, the membranes were washed and incubated for 1 h with a secondary antibody conjugated with peroxidase. (1:5000/1:10,000). Immunoreactive bands were visualized using the enhanced chemiluminescence detection

Figure 1. suPAR regulation of cell growth. DU145 cells, cultured in serum-free medium, were treated with suPAR (1.0-10 nM) for 48 h. At the end of the treatments, cells were harvested and counted using a haemocytometer (A). [3H]thymidine incorporation on DU145 cells cultured in serum-free medium and incubated for 24 h with graded doses of suPAR (1.0-10 nM). Cell number is expressed as a percentage of [3H]thymidine incorporation (B). The results of three separate experiments are presented as the mean ± SD. Cell cycle analysis of DU145 cells treated with suPAR (C and D). The adherent DU145 cells were treated for 24 h with suPAR (10 nM) in serum-free medium. All cells were then measured on a FACScan flow cytometer with an argon laser at 488 nm for excitation and analyzed using Cell Quest software. The results were expressed as the percentage of cell death by apoptosis induced by suPAR treatment. Each experiment was performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Tukey’s multiple comparison tests. *p<0.05 vs controls was considered statistically significant.
kit reagents (ECL Plus Western blotting detection system, Amersham Biosciences, UK).

**Results**

**suPAR regulation of cell growth.** We undertook experiments to examine the potential role of suPAR in regulating cell growth in PCa DU145 cells. First of all, DU145 cells, cultured for 48 h with suPAR (1.0-10 nM), were harvested and counted using a haemocytometer. As shown in Fig. 1A, suPAR, at the dose of 10 nM, inhibited the proliferation of DU145 cells. Moreover, a 24-h treatment with 10 nM suPAR significantly decreased [3H]thymidine incorporation of DU145 cells with respect to untreated cells (Fig. 1B). FACScan analysis of DU145 cells treated for 24 h with suPAR (10 nM) indicated that 14.87±1.44% of cells were in the sub-G1 phase, which is an indicator of apoptosis. The results, expressed as the percentage of death by apoptosis induced by suPAR treatment, show that suPAR increased the number of apoptotic cells from 2 to 18%.

**Effects of suPAR on cell migration and Matrigel™ invasion.** To test the effects of suPAR on DU145 cell migration and invasion, we used haptotaxis assay and Matrigel invasion chamber analysis. Fig. 2A shows that DU145 cells responded to suPAR, decreasing the capacity to migrate in response to laminin used as a haptotactic stimulus. In particular, exposure to 10 nM suPAR reduced the migratory activity of DU145 of 41%. The effect of suPAR was then evaluated on the ability of DU145 to invade a tridimensional matrix of Matrigel. Fig. 2B indicates that 10 nM suPAR exposure significantly inhibited the invasiveness of DU145 cells (47% decrease) through the Matrigel barrier.

**The effect of suPAR on ERK and FAK activation.** To clarify whether suPAR might modify ERK and FAK activation in PCa cells, we performed immunoblotting on extracts of DU145 cells exposed to either suPAR (10 nM), or CsuPAR (10 nM) for 5 min. The cell extracts were subjected to immunoblot analysis to detect ERK1/2 (A) and FAK (B) phosphorylation. Blots are representative of 3 different experiments.

**Effects of suPAR on cell migration and Matrigel™ invasion.** To test the effects of suPAR on DU145 cell migration and invasion, we used haptotaxis assay and Matrigel invasion chamber analysis. Fig. 2A shows that DU145 cells responded to suPAR, decreasing the capacity to migrate in response to laminin used as a haptotactic stimulus. In particular, exposure to 10 nM suPAR reduced the migratory activity of DU145 of 41%.

The effect of suPAR was then evaluated on the ability of DU145 to invade a tridimensional matrix of Matrigel. Fig. 2B indicates that 10 nM suPAR exposure significantly inhibited the invasiveness of DU145 cells (47% decrease) through the Matrigel barrier.

**The effect of suPAR on ERK and FAK activation.** To clarify whether suPAR might modify ERK and FAK activation in PCa cells, we performed immunoblotting on extracts of DU145 cells exposed for 2, 5 and 20 min to suPAR (10 nM). Fig. 3 demonstrates that, in our experimental conditions, both ERK and FAK were activated in untreated DU145 cells. Moreover, suPAR actually decreased ERK phosphorylation; this effect was clearly evident after 5 min of treatment (Fig. 3A). At the same time of observation, soluble uPAR (1.0-10 nM) was able to decrease, in a dose-dependent manner, ERK phosphorylation;
the inhibition of ERK activation was consistently observed with 10 nM suPAR (Fig. 3B). Then, we studied the effect of treatment for 2, 5, and 20 min with suPAR (10 nM) on FAK phosphorylation in DU145 cells (Fig. 4). Immunoblot analysis of DU145 extracts also evidenced that suPAR produced a decrease of FAK phosphorylation after 2 min of treatment, and the effect was maintained for up to 20 min.

The cleavage of the protease sensitive region of uPAR between D1 and D2 produces a short fragment (D2D3), which no longer binds uPA (41). DU145 cells exposed for 5 min to suPAR cleaved by chymotrypsin (CsuPAR) did not show the decrease of ERK and FAK phosphorylation previously described after suPAR treatment (Fig. 5A and B).

**Discussion**

The present study was aimed at investigating whether soluble uPAR is able to inhibit proliferation and/or invasion of PCa cancer cells. In particular, we performed experiments on the human prostate cancer cell line DU145, an androgen-independent PCa cell line expressing an efficient uPA-uPAR signalling system (42,44). We report, for the first time, evidence demonstrating that soluble uPAR may antagonize cell growth, promotes apoptosis and decreases both migration and Matrigel invasion of PCa cells.

The exogenous addition of recombinant suPAR significantly decreases, in a dose-dependent manner, cell proliferation and promotes apoptosis in DU145 cells. Since the activation of uPAR by uPA triggers DU145 cell proliferation (reviewed in ref. 14), one possible mechanism of action of suPAR may be related to its capability to bind uPA, subtracting the endogenous serino-protease from its membrane receptor on tumour cells. In accordance with our data, other authors (38,39,45) demonstrated a suppression of *in vivo* tumour growth and metastasis of both MDA-MB-231 human breast carcinoma and ovarian cancer OV-MZ-6/8 cells overexpressing suPAR.

The activation of the PA system is known to promote different processes that involve cell migration, which are a prerequisite for cell invasion and the establishment of metastasis. Another biological effect of suPAR reported in the present study on PCa cells was shown by *in vitro* migration and invasion assays. We demonstrated that soluble uPAR is able to influence the invasive activities of DU145 cells, since it substantially decreases the capacity to migrate in response to a haptotactic stimulus and the ability of the cells to invade a tridimensional matrix of Matrigel.

It has been reported that the uPA/uPAR complex activation is coupled to a number of intracellular pathways including extracellular signalling regulated kinase (ERK). ERK activation occurs in response to uPAR fractional occupancy, accounting for the role of uPAR autocrine signalling in determining the basal level of activated ERK in many cells (46,47). Moreover, elevated focal adhesion kinase (FAK) expression has been observed in different human cancer cell lines with increasing tumorigenic potential (48-51) and FAK was found to be phosphorylated upon uPA binding to uPAR in several cell types (17). Our data sustain the observation that, in DU145 cells, both ERK1/2 and FAK are activated in standard conditions; moreover, the effects of suPAR on the proliferation and migration of DU145 cells may be linked to a decrease of ERK1/2 and FAK phosphorylation.

The cleavage by chymotripsin prevents the inhibitory action of suPAR on ERK1/2 and FAK activation in DU145 cells and suggests different actions for suPAR and CsuPAR; furthermore, it can be suggested that the breaking of suPAR by proteases represents a mechanism by which cancer cells convert an inhibitory molecule (suPAR) into an ineffective compound (CsuPAR). To demonstrate that the action of suPAR in DU145 cells is linked to the capability of suPAR to scavenge uPA, we performed experiments on LNCaP cells. In these PCa cells, uPA is not expressed at a sufficient level to establish autocrine uPA/uPAR signalling and, as a result, suPAR is unable to affect LNCaP cell proliferation. By contrast, when we cultured...
LCaP cells on fibronectin-coated plates, an experimental condition that stimulated uPA expression (43), suppress significantly decreased cell growth. Our data are in agreement with the observations of Jo et al (19); these authors reported that, in uPAR-deficient cells (uPAR−/− murine fibroblast or human embryonic kidney 293 cells), both suPAR and CsuPAR function as a partial signalling agonist that activates ERK. On the contrary, in cells with a potent autocrine uPA-uPAR signalling system (MDA-MB 231 breast cancer cells) suPAR decreased ERK activation and inhibited proliferation, while CsuPAR was ineffective.

In accordance with the findings of other authors (19,38,39,45,52), we propose that suPAR produces different effects on PCa cell proliferation depending on the expression of the endogenous uPA-uPAR system; moreover, we suggest that intact suPAR may scavenge uPA and prevent uPA binding to membrane-anchored uPAR, producing a decrease in the level of activated ERK and FAK in PCa cells.

Prolonged uPAR suppression produces tumour cell ‘dormancy’, a dormant state during which tumour cells are present but not seemingly biologically or clinically active (reviewed in ref. 53). An increase of uncleaved suPAR able to bind endogenous uPA may be important to control proliferation and migration of cancer cells.

Preliminary studies performed in our laboratory suggest that drugs currently used for the therapeutic treatment of PCa, such as GnRH analogues, seem to counteract the effect of endogenous uPA through the increase of soluble uPAR in the conditioned media of DU145 cells (unpublished data). Even if further studies are needed to clarify the role of suPAR in controlling PCa growth and invasion, the molecular mechanism through which suPAR participates in the biology of advanced PCa cells evidenced in our studies by mechanisms unrelated to plasminogen activation, is novel and entirely untreated and may bear relevance for the long-term goal to identify new therapeutic targets aimed to trigger tumour dormancy in vivo.

Acknowledgments

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References


