Urokinase induces proliferation of human ovarian cancer cells: characterization of structural elements required for growth factor function

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Abstract Ovarian cancer metastasis is associated with an increase in the urokinase-type plasminogen activator (uPA) and its receptor uPAR. We present evidence that binding of uPA to uPAR provokes a mitogenic response in the human ovarian cancer cell line OV-MZ-6 in which endogenous uPA production had been significantly reduced by stable uPA 'antisense' transfection. High molecular weight (HMW) uPA, independent of its enzymatic activity, produced an up to 95% increase in cell number concomitant with 2-fold elevated [^{3}H]thymidine incorporation as did the catalytically inactive but uPAR binding amino-terminal fragment of uPA, ATF. uPA-induced cell proliferation was significantly decreased by blocking uPA/uPAR interaction by the monoclonal antibody IIIF10 and by soluble uPAR. The efficiency of the uPAR binding synthetic peptide cyclo^{19,31}uPA$_{19-31}$ to enhance OV-MZ-6 cell growth proved this molecular domain to be the minimal structural determinant for uPA mitogenic activity. Dependence of uPA-provoked cell proliferation on uPAR was further demonstrated in Raji cells which do not express uPAR and were thus not induced by uPA. However, upon transfection with full-length uPAR, Raji cells acquired a significant growth response to HMW uPA and ATF.

Key words: Ovarian cancer; Urokinase-type plasminogen activator; Urokinase-type plasminogen activator receptor; Mitogen; Cell proliferation; Antisense transfection

1. Introduction

The serine protease urokinase-type plasminogen activator (uPA) is synthesized and secreted by normal and malignant cells and is implicated in a variety of physiological and pathophysiological processes including extracellular matrix turnover, tumor cell migration, and invasion [1]. The protease uPA also exerts biological effects characteristic for molecules with signal transducing properties including chemotaxis, migration, adhesion, and mitogenesis [2]. Mitogenic activity of uPA has been observed for several cell types in a highly cell-type-specific manner with still inconclusive reports on the structural and molecular requirements [3–11]. Pro-uPA is a multi-domain proenzyme which encompasses three functionally autonomous domains: the amino-terminal growth factor-like domain (GFD) containing the binding site for the uPA receptor (uPAR, CD87), the kringle domain, and the serine protease region. Pro-uPA is converted into proteolytically active high molecular weight (HMW) uPA by the action of certain proteases, e.g. plasmin, HMW kallikrein, cathepsin B and L, and nerve growth factor-γ [1]. Upon release of the amino-terminal fragment (ATF = GFD plus kringle domain), HMW uPA is converted into a still catalytically active form of uPA, LMW uPA, lacking the uPAR binding site. On cell surfaces, uPA interacts with a specific glycosylphosphatidylinositol (GPI)-anchored high affinity receptor, uPAR. Upon binding to uPAR, uPA retains its full catalytic activity thus converting plasminogen into plasmin [12,13]. The activity of uPA is controlled by plasminogen activator inhibitors type 1 and type 2 [1].

In recent years several studies have been undertaken to identify prognostic factors to predict disease-free and overall survival of patients afflicted with solid human cancer. A strong statistically independent prognostic impact has been attributed to uPA and its inhibitor PAI-1 in a variety of malignancies [1] including ovarian cancer [14]. The aim of the present study was to investigate whether uPA stimulates cell proliferation in ovarian cancer cells and to characterize the structural elements within the uPA molecule required for growth factor-like function.

2. Materials and methods

2.1. Materials

Human HMW uPA obtained from Curasan Pharma GmbH (Kleinostheim, Germany) was further purified by FPLC gel using a Hi-Load 16/60 Superdex 15/75 column (Pharmacia Biotech, Freiburg, Germany). Inactivation of HMW uPA was achieved with 4×10^{-4} M Pefabloc SC (Pentapharm AG, Basel, Switzerland) or 4×10^{-6} M H-Glu-Gly-Arg-CH_2Cl (Bachem Biochemica, Heidelberg, Germany). LMW uPA was purchased from Serono, Munich, Germany; human ATF from American Diagnostica (Greenwich, CT, USA). The uPA-related peptides cyclo^{19,31}uPA$_{19-31}$ and cyclo^{20,30}uPA$_{20-31}$ (V20C; W30C, C31W) have been described earlier [15]. uPA, PAI-1, uPAR, and D-dimer concentrations were determined by ELISA kits Imubind uPA # 894, Imubind PAI-1 # 821, Imubind uPAR # 893, and D-dimer ELISA, respectively (American Diagnostica). Solid phase uPA ligand binding assays were performed as published [16]. Recombinant soluble uPAR (uPAR$_{1-277}$) expressed in Chinese hamster ovary (CHO) cells was purified from cell culture supernatants by uPA ligand affinity chromatography and its uPA binding activity assessed by flow

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cytofluorometry [17]. The monoclonal antibodies (mAb) IIIF10 and IID7 directed against uPAR were generated as described [18]. [^{35}S]Translabel (methionine/cysteine) and [^{3}H]thymidine were purchased from ICN, Eschwege, Germany; phosphoinositol-specific phospholipase C (PI-PLC) was from Boehringer Mannheim, Mannheim, Germany.

2.2. Establishment of two cell systems to study the mitogenic activity of uPA

2.2.1. Human ovarian cancer cells. The human ovarian cancer cell line OV-MZ-6 [19,20] exhibits a broad range of clonal heterogeneity regarding the expression and protein synthesis of individual cell clones, including that of uPA. Prior to uPA stimulation we suppressed endogenous uPA expression by stable uPA 'antisense' transfection. The OV-MZ-6 cell line was subcloned and three OV-MZ-6 cell clones selected exhibiting uPA, uPAR, and PAI-1 antigen content similar to the non-clonal wild type cell line. Tumorigenicity of these clones was confirmed in BALB/c nude mice as described [21]. Clone 8 was chosen and subjected to stable uPA 'antisense' transfection using lipofectin. A uPA cDNA fragment of 647 bp length was cloned in 'antisense' orientation into the expression plasmid pRc-RSV (Invitrogen, San Diego, CA, USA). Transfections using pRc-RSV with the identical insert in 'sense' orientation or the empty plasmid alone ('vector') served as controls. Several OV-MZ-6 cell clones from each transfection were isolated upon neomycin selection and tested for expression of uPA, PAI-1, and uPAR, respectively.

2.2.2. Raji cells. Lymphoblastoid Raji cells were cultivated in RPMI 1640 medium (Gibco, Eggenstein, Germany), 10% (v/v) fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Transfection of Raji cells with full-length uPAR cDNA cloned into pRc-RSV was performed by electroporation (Bio-Rad Gene Pulser, Munich, Germany) at 250 V and 960 μF. Stably transfected cell clones were isolated upon selection with neomycin and purified to clonality through five rounds of limited dilutions.

2.3. Treatment of OV-MZ-6 cell with PI-PLC

In order to release GPI-anchored proteins including uPAR from the tumor cell surface [22], prior to uPA stimulation, OV-MZ-6 cell monolayers were treated with 5 μg/ml bacterial PI-PLC in PBS, pH 7.4, for 2 h at 37°C and washed in PBS thereafter.

2.4. Evaluation of cell proliferation upon uPA stimulation

Cells were cultivated for 24 h in medium containing 1% (v/v) FCS, a medium in which also uPA stimulation was performed. For the evaluation of cell proliferation three experimental approaches were chosen:(a) cell counting, (b) [^{3}H]thymidine incorporation, and (c) S-phase determinations. (a) Cells were counted in a Neubauer hemocytometer upon Trypan blue exclusion. (b) [^{3}H]Thymidine incorporation was determined as described earlier [23]. At certain time intervals after the addition of uPA, OV-MZ-6 cells were pulsed in FCS-free medium for 1 h with 5 μCi of [^{3}H]thymidine, washed in PBS, detached with 1% (w/v) EDTA, and precipitated with 10% (v/v) trichloroacetic acid (TCA) for 30 min. The precipitate was washed in PBS and the TCA-insoluble fraction dissolved in 1 ml of 0.1 N NaOH, 1% (w/v) SDS. After liquid scintillation counting counts per minute (cpm) per cell were evaluated. (c) Determination of S-phase fraction was performed according to Harbeck and coworkers [24].

2.5. In vitro invasion and fibrin degradation assays

Measurements of in vitro invasion were conducted in a double-filter sandwich assay system as described [25] using metabolically labeled cells [20]. Fibrin matrix degradation assays were performed with individual, stable OV-MZ-6 transfectants as described earlier [20]. Crosslinked fibrin degradation products including D-dimers were quantified by ELISA.

3. Results

3.1. Effect of uPA 'antisense' transfection on uPA content and invasion of OV-MZ-6 cells

Stable uPA 'antisense' transfection reduced uPA secretion into cell culture supernatants up to 40-fold while uPA 'sense' and 'vector' OV-MZ-6 transfectants exhibited uPA synthesis and secretion rates similar to the parental cell line (clone 8) (Fig. 1). uPAR and PAI-1 levels remained unaltered. Concomitant with suppressed uPA synthesis, OV-MZ-6 uPA 'antisense' transfectants exhibited a pronounced, up to 30% decrease in their invasive capacity and a significantly reduced fibrinolytic activity (data not shown). In contrast, uPA 'sense' and 'vector' OV-MZ-6 transfectants behaved like non-transfected cells.

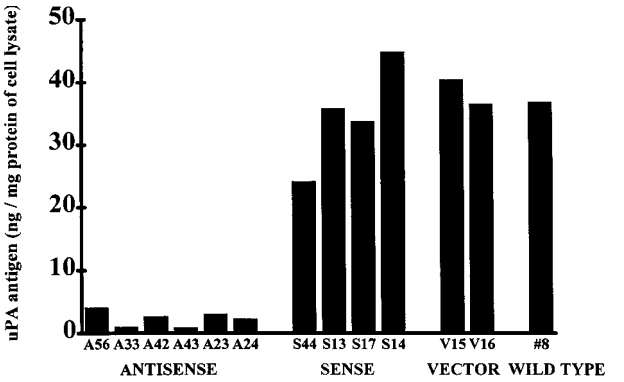

Fig. 1. Quantification of uPA antigen content in culture supernatants of OV-MZ-6 transfectants. OV-MZ-6 cell clones were transfected with uPA 'antisense' and uPA 'sense' encoding expression plasmids as described and screened for uPA expression in cell culture supernatants. Transfections with the plasmid without insert ('vector') and untransfected wild type OV-MZ-6 cells, respectively, served as controls. uPA antigen content is given as ng/mg protein of cell lysates.

3.2. Effect of uPA on OV-MZ-6 cell proliferation

In principle, a series of different ovarian carcinoma cell lines including OV-MZ-6, OV-MZ-13, OV-MZ-19, and OVCAR-3 [19] responded to HMW uPA with increases in cell number. As test cells we chose the tumorigenic cell line OV-MZ-6 with significantly reduced uPA expression due to uPA 'antisense' transfection (see Section 2.2). Addition of HMW uPA to OV-MZ-6 uPA 'antisense' transfectants increased cell proliferation in a concentration-dependent manner. Enhancement of cell proliferation was maximal (plus 94% ± S.D. 12%) at a uPA concentration of about 1 nM within 96 h compared to unstimulated controls. Higher concentrations of uPA led to a gradual decrease of the uPA stimulatory effect, an observation also made in other ligand/receptor systems (Fig. 2). Similar to increases in OV-MZ-6 cell number, [^{3}H]thymidine incorporation was about 2-fold induced by 1 nM HMW uPA within 96 h (data not shown). Determination of cell cycle fractions revealed that in uninduced OV-MZ-6 cells, S-phase fraction gradually declined within 96 h from 19.8 to 9.5% when cells were cultured in DMEM containing 1% (v/v) FCS. However, as soon as HMW uPA was added to cell culture supernatants, the decline of S-phase fraction was prevented. Thus after 96 h an about 2.5-fold higher S-phase fraction was found in uPA-compared to non-stimulated cells. Concomitantly, uPA stimulation led to an up to 15% decrease of the G0/G1 fraction of the cell cycle. When HMW uPA was inactivated by either Pefabloc or H-Glu-Gly-Arg-CH_2Cl it was still capable of inducing cell proliferation.

3.3. Structural requirements for the mitogenic activity of uPA

In order to define the structural elements within the uPA molecule responsible for its cytokine-like activity, OV-MZ-6

uPA 'antisense' transfectants were exposed to different molecular forms of uPA (HMW uPA, LMW uPA, ATF) in equimolar concentrations. Exposure of OV-MZ-6 cells to ATF (Fig. 3) or the ATF fusion proteins ATF-HSA [26] and ATF-UTI [27] (data not shown) produced similar increases in cell number as did HMW uPA (plus 69% ± 9.6). HMW uPA and ATF also provoked a 2-fold elevated [^{3}H]thymidine incorporation (data not shown). Catalytically active LMW uPA devoid of the uPAR binding region did not exhibit mitogenic activity (Fig. 3) and did also not alter the rate of [^{3}H]thymidine incorporation. Thus uPA binding to uPAR was required for mitogenic activity of uPA. In order to determine the minimal structural elements for mitogenic activity, OV-MZ-6 uPA 'antisense' transfectants were exposed to the synthetic uPA-derived cyclic peptide cyclo^{19,31}uPA$_{19-31}$ encompassing the uPAR binding region of uPA. The peptide cyclo^{19,31}uPA$_{19-31}$ induced a significant mitogenic response in OV-MZ-6 uPA 'antisense' transfectants whereas the uPA-related control peptide cyclo^{20,30}uPA$_{20-31}$ (V20C; W30C, C31W), which does not bind to uPAR [15], was not effective (Fig. 3).

3.4. Effect of blocking uPA/uPAR interaction on uPA-induced cell proliferation

The monoclonal antibody mAb IIIF10 (8 μg/ml) [18] directed against the uPAR binding region of uPA was added to OV-M-6 uPA 'antisense' transfectants 2 h prior to uPA stimulation followed by repeated additions of the same amount of mAb IIIF10 every 24 h. Within 96 h mAb IIIF10 almost completely abrogated uPA-mediated growth-promoting activity (Fig. 3). MAb IID7 (directed against domain II of uPAR) which does not interfere with uPA binding was ineffective (Fig. 3). The addition of recombinant soluble uPAR (CHO-uPAR$_{1-277}$) as a 'scavenger' for uPA [17] during the uPA stimulation period of OV-MZ-6 cells also resulted in a significantly suppressed mitogenic cell response (Fig. 3).

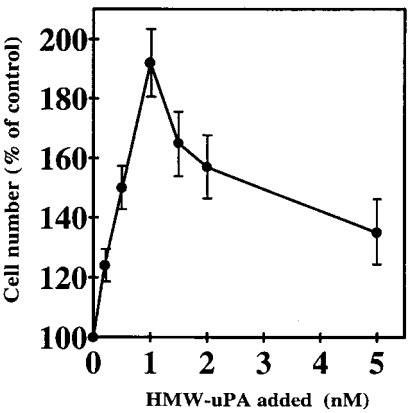

Fig. 2. Concentration-dependent effect of HMW uPA on OV-MZ-6 cell proliferation. OV-MZ-6 uPA 'antisense' transfectants were exposed to increasing concentrations of HMW uPA of 0.2–5 nM in DMEM, 1% (v/v) FCS and incubated for 96 h. Values are given as cell number in % setting unstimulated controls at 100%. At least five independent experiments were performed in duplicate; vertical bars indicate the standard error of the means.

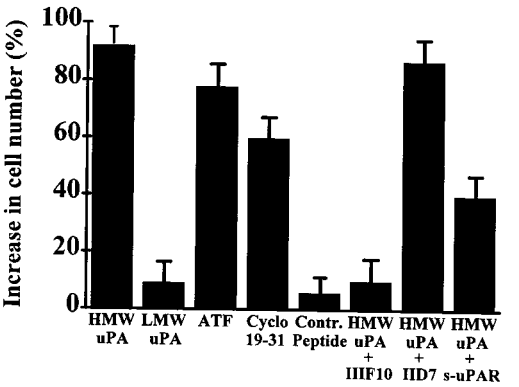

Fig. 3. Structural requirements for the mitogenic effect of uPA. OV-MZ-6 uPA 'antisense' transfectants were treated prior to uPA stimulation as described. HMW uPA, LMW uPA, or ATF were added in equimolar concentrations (1 nM) in DMEM, 1% FCS (v/v). The uPA-derived synthetic peptide cyclo^{19,31}uPA$_{19-31}$ was added at 20 μg/ml based on solid phase uPA ligand binding assays [17]. The control peptide cyclo^{20,30}uPA$_{20-31}$ (V20C; W30C, C31W) which did not exhibit uPAR binding activity served as control. Blockade of uPA/uPAR interaction was further achieved by mAb IIIF10. The mAB IID7 which does not interfere with uPA binding to uPAR served as control. In addition, soluble uPAR (s-uPAR) was coincubated with HMW uPA at a concentration of 3 μg/ml. Cell numbers were evaluated by cell counting within 96 h; the values are given as increases in cell number in %. Five independent experiments were performed in duplicate; vertical bars indicate the standard error of the means.

3.5. Growth-promoting effect of uPA in Raji cells

In order to prove the dependence of the cell growth-promoting effect of uPA on its interaction with uPAR we investigated its mitogenic activity in Raji cells which do not express uPAR. Raji cells were exposed to HMW uPA, LMW uPA, or ATF, and cells were counted after 96 h. Raji cells did not respond to either molecular form of uPA with increases in cell number. However, as soon as Raji cells were stably transfected with an expression plasmid encoding uPAR, significant responsiveness of Raji cells to HMW uPA (Fig. 4) was acquired resulting in an approximately 40% increase in cell number. As seen in human ovarian cancer cells, ATF produced a similar proliferative response of Raji cells whereas LMW uPA was not effective (data not shown).

3.6. Effect of uPAR shedding by PI-PLC on uPA-mediated growth stimulation

Treatment of OV-MZ-6 uPA 'antisense' transfectants with PI-PLC, known to release GPI-anchored proteins such as uPAR from cell surfaces [22], prior to uPA stimulation resulted in a complete loss of uPA-triggered growth responses (data not shown) accompanied by an up to 7-fold increase of soluble uPAR in cell culture supernatants. The fact that PI-PLC treatment in non-stimulated cells resulted in a moderately weaker growth promotion compared to non-stimulated cell which had not been treated by PI-PLC is possibly due to an additive but weak growth stimulation of cells by autocrinely secreted uPA. Release of uPAR by PI-PLC can thus lead to a further minimal decline in cell growth even in cells which had not been stimulated by uPA. We also cannot rule out that other GPI-anchored receptors might be involved in growth stimulation of cells and are lost upon PLC treatment.

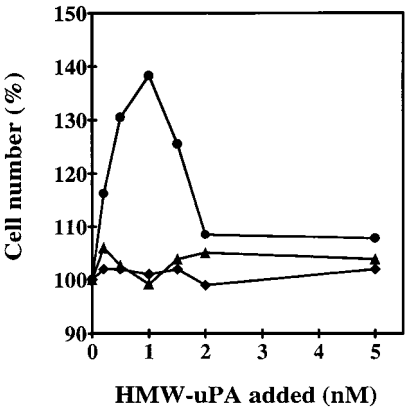

Fig. 4. Mitogenic effect of uPA on wild type and uPAR-transfected Raji cells. Lymphoblastoid Raji cells devoid of detectable uPAR expression levels were stably transfected with an expression plasmid encoding uPAR and exposed to 1 nM of HMW uPA (●). HMW uPA stimulation was also performed with wild type Raji cells (▲) and Raji cells which had been transfected with the empty expression vector only (◆). Cell number was evaluated by cell counting after 96 h. Values are given as cell number in % by setting values from unstimulated controls at 100%. Three independent experiments were performed; a typical result is shown.

4. Discussion

Extensive efforts have been undertaken to explore biological features of uPA triggering intracellular signal transduction, involved in cell proliferation [3–11], cell adhesion, cell migration, and chemotaxis [2]. Primary tumor growth and the establishment of metastases require rapid cell proliferation and the involvement of growth factors. In recent years a mitogen-like function has been described for uPA which appears to be highly cell-type-restricted and cell-type-specific regarding the requirement of structural elements within the uPA molecule. In the present investigation we studied for the first time the mitogenic activity of uPA in different human ovarian cancer cells. The increase in cell number was most pronounced in tumorigenic OV-MZ-6 uPA 'antisense' transfectants with a maximal stimulatory effect at a uPA concentration of about 1 nM. HMW uPA, ATF as well as ATF fusion proteins [26,27] exhibited comparable growth factor-like activity. At higher concentrations of HMW uPA a subsequent decline of the uPA-mediated growth induction was noticed, a phenomenon which was also observed in other ligand/receptor systems and is discussed to be due to receptor desensitization.

In osteosarcoma cells proliferative responses to both HMW uPA and ATF had been demonstrated before, however, depending on an unaltered glycosylation pattern of uPA. Non-glycosylated recombinant HMW uPA expressed in *Escherichia coli* did not produce a proliferative cell response [7]. Moreover, a special post-translational modification, a single fucosylation site at threonine-18 of uPA, seemed to be required [28]. In human ovarian carcinoma cells, however, we showed that glycosylation was not required for stimulation of cell proliferation since addition of recombinant ATF, which is non-glycosylated due to its production in *E. coli*, was also effective. In contrast to our findings, ATF did not induce cell proliferation in smooth muscle cells [11] and in human breast cancer cells [10].

In human ovarian cancer cells we proved that the mitogen-like function of uPA was independent of its enzymatic activity by pretreating HMW uPA with specific active site blockers. Inactivation of HMW uPA did not alter its mitogenic activity. In the human epidermal tumor cell line CCL 20.2 [3,4] and GUBSB melanoma cells [5], however, it had been demonstrated before that uPA-induced cell proliferation was only effective when both aspects, uPAR binding and enzymatic activity of uPA, were fulfilled. In the latter study, consequently, cell proliferation could be significantly reduced by specific antibodies directed against uPA inhibiting the uPA/uPAR interaction. Also, monoclonal antibodies directed against the catalytic center of uPA markedly decreased [^{3}H]thymidine incorporation [5].

The necessity of uPAR occupation for cytokine-like activity of uPA in human ovarian cancer cells independent of enzymatic activity was verified by us in another cell system, lymphoblastoid Raji cells which do not express uPAR on their cell surface. Wild type Raji cells did not respond to uPA stimulation; however, in accordance with our findings in human ovarian cancer cells, they did acquire responsiveness to HMW uPA and ATF upon transfection with an uPAR cDNA-encoding expression plasmid. These data further support the notion of a strong dependence of the growth factor-like activity of uPA on its binding to uPAR which is in line with the finding that uPAR antibodies blocking uPA/uPAR interaction [18] abrogated the mitogenic activity of uPA. Reduction of growth factor activity of uPA was also achieved by the addition of soluble recombinant uPAR as 'scavenger' for uPA [17] as well as by shedding uPAR upon PI-PLC treatment of cells [22].

In order to further narrow down the structural elements within the uPA molecule required for triggering signal transduction, we exposed human ovarian cancer cells to the recombinant uPA-derived synthetic peptide cyclo^{19,31}uPA$_{19-31}$ spanning the uPAR binding region [15]. The peptide cyclo^{19,31}uPA$_{19-31}$ significantly promoted OV-MZ-6 cell proliferation thus pointing to the uPAR binding region as the minimal structural determinant within the uPA molecule.

Current research aims at establishing the molecular basis of the uPA/uPAR signaling pathway as well as transmembrane molecules interacting with GPI-anchored uPAR. Integrins might be candidates in concert with intracellular *src* protein tyrosine kinases [29,30] leading to the phosphorylation of certain proteins [31,32]. In ovarian carcinoma cells a rapid and transient c-*fos* gene induction by uPA had been noticed [33]. Moreover, in another human ovarian carcinoma cell line, IGR-OV1, it had been demonstrated that uPA/uPAR-dependent cell migration is associated with tyrosine kinase activation [34].

In conclusion, our data suggest that uPA might constitute an autocrine uPAR-mediated growth-promoting stimulus on cell surfaces thereby contributing to the rapid growth characteristics of malignant cells. Indeed, aggressively growing tumor cells very often produce significantly elevated levels of uPA compared to less aggressive or normal cells [1]. Thus targeting uPA might be effective not only to reduce tumor cell invasion but also to provoke an anti-proliferative effect within cancer therapy.

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