Evidence of a non-conventional role for the urokinase tripartite complex (uPAR/uPA/PAI-1) in myogenic cell fusion

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SUMMARY

Urokinase can form a tripartite complex binding urokinase receptor (uPAR) and plasminogen activator inhibitor type-1 (PAI-1), a component of the extracellular matrix (ECM). The components of the tripartite complex are modulated throughout the in vitro myogenic differentiation process. A series of experiments aimed at elucidating the role of the urokinase tripartite complex in the fusion of human myogenic cells were performed in vitro. Myogenic cell fusion was associated with increased cell-associated urokinase-type plasminogen activator (uPA) activity, cell-associated uPAR, and uPAR occupancy. Incubation of cultures with either uPA anticalytic antibodies, or the amino-terminal fragment of uPA (ATF), which inhibits competitively uPA binding to its receptor, or anti-PAI-1 antibodies, which inhibit uPA binding to PAI-1, resulted in a 30 to 47% decrease in fusion. Incubation of cultures with the plasmin inhibitor aprotinin did not affect fusion. Decreased fusion rates induced by interfering with uPAR/uPA/PAI-1 interactions were not associated with significant changes in mRNA levels of both the myogenic regulatory factor myogenin and its inhibitor of DNA binding, Id. Incubation of cultures with purified uPA resulted in a decrease in fusion, likely due to a competitive inhibition of PAI-1 binding of endogenous uPA. We conclude that muscle cell fusion largely depends on interactions between the members of the urokinase complex (uPAR/uPA/PAI-1), but does not require proteolytic activation of plasmin. Since the intrinsic muscle cell differentiation program appears poorly affected by the state of integrity of the urokinase complex, and since cell migration is a prerequisite for muscle cell fusion in vitro, it is likely that the urokinase system is instrumental in fusion through its connection with the cell migration process. Our results suggest that the urokinase tripartite complex may be involved in cell migration in a non conventional way, playing the role of an adhesion system bridging cell membrane to ECM.

Key words: Myogenic cell fusion, Cell migration, Cell adhesion, Plasminogen activator system, Urokinase

INTRODUCTION

Both gene activation and cellular environment play important roles in the control of cell proliferation and differentiation (Mareel et al., 1993; Adams and Watt, 1993; Ingber, 1993; Watt et al., 1994; Breton et al., 1995). The cellular environment may be modified by proteinases and their inhibitors, such as the plasminogen activation system (PAs) that leads to formation of plasmin, a wide-spectrum proteinase. The plasminogen activation system (PAs) includes the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA). tPA is mainly involved in fibrinolysis, while uPA has been implicated in tissue remodeling, cell migration, and neoplasia (Meissauer et al., 1991; Pepper and Montesano, 1991; Pöllänen et al., 1991; Stahl and Mueller, 1994; Sumiyoshi et al., 1991).

The urokinase system includes urokinase receptor (uPAR), urokinase (uPA) and two specific inhibitory molecules (PAI-1 and PAI-2) (Andreasen et al., 1990; Ragno et al., 1993). uPA is a serine proteinase that acts on the ECM through the cleavage of plasminogen to plasmin, an active ECM proteolytic factor (Quigley et al., 1990; Quax et al., 1992a; Fazioli and Blasi, 1994), and by direct proteolysis of ECM components such as fibronectin (Quigley et al., 1987; Gold et al., 1992).

In addition to its proteolytic effects, there is an increasing evidence that uPA is involved in non-proteolytic signaling (Wang et al., 1995; Resnati et al., 1996), operative in differentiation and adhesion processes (Waltz and Chapman, 1994; Dumler et al., 1993). Such non-conventional effects of urokinase may depend on fine interactions within the tripartite complex formed by uPAR, uPA, and the PAI-1 inhibitor (Waltz and Chapman, 1994). Recently, it was shown that uPAR binds to vitronectin (Wei et al., 1994) and that PAI-1 inhibits this interaction (Kanse et al., 1996).
We have previously demonstrated that PAs components are present in extracts of both rat and human myogenic satellite cells (Le Moigne et al., 1990; Quax et al., 1992b), and that their level and activity are modulated throughout the myogenic differentiation process in vitro (Quax et al., 1992b). We also showed that uPAR mediates mechanical force transfer to myogenic cells (Wang et al., 1995). Both uPAR-dependent cytoskeletal remodeling (Wang et al., 1995), and adhesion properties of the urokinase system on ECM (Waltz and Chapman, 1994; Wei et al., 1994; Bussolino et al., 1994), are likely to play an important role in cell migration.

We present, herein, an in vitro study aimed at elucidating the role of the urokinase system in cell differentiation process. We used primary cultures of human myogenic cells, a model that encompasses cell proliferation, migration, fusion, and differentiation dependent gene activation. Our results provide evidence for an important but non-conventional role for the urokinase system.

**MATERIALS AND METHODS**

**Myogenic cell culture determinations**

**Cell culture**

Primary human muscle cell cultures were grown from leg muscles of normal patients undergoing orthopaedic surgery obtained after informed consent as previously described (Quax et al., 1992b; Barlovaz-Meimon et al., 1994). For all experiments first passage cell cultures were used. The extent of contamination by non myogenic cells was assessed by immunocytochemistry for desmin, a specific marker of the myogenic lineage (Lazarides and Hubbard, 1976), using indirect immunofluorescence assay (monoclonal antibody and TRITC-antibody conjugate from Sigma).

For all experiments cells were plated at 2,000 cells per square centimeter, respectively, in 1.9 cm² dishes and 25 cm² flasks for proliferation, differentiation, uPA and creatine phosphokinase (CPK) activity studies and for mRNA studies.

**Determination of myogenic cell proliferation and fusion rates**

Proliferation and fusion rates were evaluated by counting trypsinized cells on a Malassez haemocytometer, and fixed, stained nuclei by the May-Grünwald-Giemsa method, respectively.

Proliferation was expressed as the number of mononucleated cells per square centimeter. Fusion of myogenic cells was assessed by immunocytochemistry for desmin, a specific marker of the myogenic lineage (Lazarides and Hubbard, 1976), using an indirect immunofluorescence assay (monoclonal antibody and TRITC-antibody conjugate from Sigma).

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**Determination of biochemical myogenic cell differentiation**

Myogenic cell differentiation was assessed biochemically by determination of muscle creatine kinase (CK) activity and CK isoforms in cell lysates using commercially available tests (Merck Granulat 2.5 plus kit, BioMerieux Enzyme @ CK NAC) as previously described (Lagord et al., 1993).

**Determination of uPA activity**

uPA activity was determined in cell lysates according to a previously described method (Drapier et al., 1979; Quax et al., 1992b) using the S-2251 Kabi (Sweden) chromogenic substrate and a commercial uPA standard (Choay 60,000 IU/mg). Protein content was determined using the Lowry method (Lowry et al., 1951). uPA specific activity was expressed as mDO/min per mg protein.

**uPAR analysis**

The presence of uPAR on cell membranes was assessed using a previously described method (Stopelli et al., 1986; Behrendt et al., 1990). Briefly, cells were washed with a binding buffer (serum free medium supplemented with 0.1% bovine serum albumin and 50 mM Hepes, pH 7.0) and incubated with iodinated amino-terminal fragment of uPA (125I-uPA). The specificity of the reaction was determined by the addition of an excess (100 nM) of unlabelled ATF. After removal of the supernatant, the cells were washed four times in buffer (PBS, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 1 mg/ml bovine serum albumin) and scraped in PBS. Radioactivity in the cell fraction was determined using a γ-counter.

The number of uPA receptors was estimated by saturating concentrations of 125I-ATF. To evaluate occupancy of uPAR, the procedure was performed with and without previous acid washing of cells which dissociates bound ligand (total uPAR: after acid washing; free uPAR: without acid washing) (Stopelli et al., 1986).

**Myogenin and Id mRNA analysis**

Myogenin mRNA, which encodes late myogenic regulatory factor (MRF), is expressed upon myotube fusion, i.e. after differentiation, whereas Id mRNAs encode Id factors that inhibit MRF activity by acting in a dominant negative manner (Rudnicki and Jaenisch, 1995). Myogenin and Id mRNA were analyzed by northern blotting. Total RNAs from mononucleated and plurinucleated myogenic cells were prepared according to the method of Chomczynski and Sacchi (1987).

The total amount of RNA isolation was determined by measuring the absorbence at 260 nm. RNA samples were analysed using electrophoresis on a 1% denaturating agarose gel containing 2.2 M formaldehyde, soaked and transferred to nylon membrane in 20× SSC (standard saline citrate; 1× SSC is 150 mM sodium chloride and 15 mM sodium citrate). The blots were hybridized with random-primed 32P-labelled cDNA overnight at 55°C in 5× SSC, 50 mM phosphate buffer (pH 7), 5× Denhardt’s, 0.1% SDS, 0.5 mM EDTA, 100 μg/ml of denatured salmon sperm DNA. Blots were then washed, three times for 10 minutes each, at room temperature in 2× SSC, 0.5% SDS, and 15 minutes at 55°C in 0.2× SSC, 0.5% SDS.

The myogenin cDNA probe was a 1.5 kb EcoRI fragment of a murine myogenin cDNA (Wright et al., 1989). The Id cDNA probe (a 0.8 kb Smal fragment of a murine Id cDNA) was kindly provided by Dr Harold Weintraub (Benezra et al., 1990). The rate of hybridization was quantified by scanning the gels using the Phosphoimager program.

**Effector assays**

All effectors were added to human myogenic cell cultures at day 4. For proliferation and fusion studies, changes were assessed up to day 10, without changing the medium from day 4 to day 10. For mRNA studies, changes were assessed at day 8 and 12 and medium was changed every 4 days. The same medium was used throughout proliferation and differentiation to avoid any effect not due to the effector itself.

**Inhibition of uPA binding to uPAR**

The amino-terminal fragment of uPA (ATF) is devoid of catalytic activity, but binds to uPAR and behaves as a competitive uPA antagonist (Stopelli et al., 1985). ATF [1-143] (from Dr Nina Pedersen) was added to cultures at a dose of 100 ng/ml. For comparison, myogenic cells were incubated with phosphatidylinositol specific phospholipase C (PI-PLC, Boehringer, Germany). PI-PLC releases glycolipid anchored surface molecules (Ferguson and Williams, 1988), including uPAR (Ploug et al., 1991), and is known to block myogenic cell fusion almost completely (Nameroff and Munar, 1976).

**Inhibition of uPA activity by uPA-anticatalytic antibodies**

An antibody to the catalytic region of uPA was purchased from...
American Diagnostica (#394, New York, USA) and added to cultures at a dose of 5 μg/ml.

Inhibition of uPA binding to PAI-1
Anti-human PAI-1 antibody was purchased from American Diagnostica (#379, New York, USA) and added to cultures at a dose of 5 μg/ml.

Control of the specificity of antibodies
A non relevant IgG (#64-335, ICN, USA) was used as control. It was added to cultures at a dose of 5 μg/ml in the same conditions as the antibodies.

Inhibition of plasmin activity by aprotinin
Plasmin inhibitor aprotinin was purchased from Sigma (#A1153, France) and added to cell cultures at high doses (1 to 50 TIU/ml).

Incubation of myogenic cells with uPA
Two purified uPA were tested. One was purchased from American Diagnostica (#128, USA) and the other one, human purified sterile uPA, was prepared by Dr Nina Pedersen. uPA was added to cultures at a dose of 5 μg/ml.

Statistical analysis
Paired and non-paired Student’s t-tests were used for statistical analysis. A P<0.05 was considered significant.

RESULTS

The three main developmental steps of human satellite cells in culture are proliferation (day 4 to day 6), alignment of the cells (day 8) and fusion, i.e. formation of plurinucleated myotubes (about day 10). To standardize results from many cultures (at least 3 for each experiment) we assigned the value 100 for the control at day 10, for proliferation, morphological and biochemical differentiation as well as uPA activity studies.

Determination of uPA activity during in vitro myogenesis
uPA activity in cell lysates increased dramatically from day 6 to day 8 of culture, day 8 being a very important point corresponding to the alignment of cells before their fusion. The increase appeared prior to the main increase of both morphological (number of nuclei included in a myotube per square centimeter) and biochemical (CPK activity) differentiation (Fig. 1).

uPAR occupancy analysis
The measure of 125I-ATF binding at various times of culture, with or without acid washing to dissociate bound ligand, showed the number of uPAR raised at time of fusion together with uPAR occupancy by uPA. At time of proliferation, free uPAR accounted approximately for 50% of total uPAR, while at time of differentiation the percentage was less than 20%. This result was reproduced in several experiments although the exact timing changed somewhat with cells from different individuals. The results in one culture are shown in Fig. 2.

Inhibition of myoblast fusion by inhibiting uPA binding to uPAR by ATF
Incubation of cultures with 100 ng/ml ATF resulted in an unchanged proliferation rate (Fig. 3) but in about 50% a decrease in fusion at day 10 (Figs 4, 5). Incubation of cultures with PI-PLC resulted in a 90% decrease in fusion (Fig. 4). ATF-induced decrease in fusion was dose-dependent (data not shown) as previously reported (Quax et al., 1992a) and time-dependent (Fig. 5). In contrast to the decrease in morphological differentiation, CPK activity increased moderately at day 10 (Fig. 6). Distribution of CPK isoforms remained unchanged (data not shown).

Inhibition of uPA activity by uPA-anticatalytic antibodies inhibits myoblast fusion
Incubation of cultures with uPA-anticatalytic antibodies (5 μg/ml) resulted in an unchanged proliferation rate (Fig. 3), a 30% decrease in fusion rate at day 10 (Fig. 4), and a non significant variation of total CK activity (data not shown).
Inhibition of PAI-1 by anti-PAI-1 antibodies inhibits fusion

Incubation of cultures with anti-PAI-1 (5 μg/ml) antibody resulted in an unchanged proliferation rate (Fig. 3), a 47% decrease in fusion at day 10 (Fig. 4), and a slight increase in total CK activity in one culture (data not shown).

Incubation with non relevant IgG

Incubation of cultures with a non specific antibody IgG1 (5 μg/ml) did not result in a change in either proliferation (Fig. 3) or fusion (Fig. 4).

Inhibition of plasmin activity by aprotinin

Incubation of cultures with the plasmin inhibitor aprotinin was associated with a slight mitogenic effect (Fig. 3), but no change in fusion (Fig. 4) at doses of 5, 10 and 20 TIU/ml. A cytotoxic effect of aprotinin on human muscle cells was observed at a concentration of 50 TIU/ml (data not shown). Neither a decrease in total CK activity nor abnormal distribution of CK isoforms was observed at doses of 5, 10 and 20 TIU/ml.

Incubation of myogenic cells with uPA

Addition of uPA (5 μg/ml) to cultures resulted in unchanged proliferation (Fig. 3) and a 33% decrease in fusion at day 10 (Fig. 4).

Myogenin and Id mRNA analysis

Northern blotting showed a non significant decrease in both myogenin and Id mRNA levels compared with controls, at day 12 of cultures incubated with ATF (Fig. 7).

DISCUSSION

In the present study, effectors preventing formation of the urokinase tripartite complex, including anti-uPA antibodies (directed against the urokinase catalytic site), ATF, a competitive inhibitor of uPA binding to its receptor, and anti-PAI-1 antibodies, markedly decreased fusion of myogenic cells. The fusion process appeared independent from plasmin, and decreased fusion rates induced by inhibiting uPAR/uPA/PAI1...
interactions, were not associated with significant changes in mRNA levels of the myogenic transcription factor myogenin, or its inhibitor Id. Addition of uPA to cell culture did not modify proliferation but decreased morphological differentiation.

The finding of increased cell-associated uPA activity and uPAR at time of myogenic cell fusion confirmed the strong increase in cell-associated uPA activity and mRNA previously documented during myogenic cell differentiation, together with modulation of mRNA levels of other components of the PAs, including uPA, tPA, and PAI-2 (Quax et al., 1992b). As with uPA activity and uPAR in the present study, the levels of all relevant mRNAs increased shortly before myoblast fusion, suggesting that plasminogen activators could play a role in the in vitro myogenic process.

PI-PLC, which cleaves uPAR and other glycolipid-anchored proteins from the cell surface, inhibited myogenic fusion dramatically, as previously reported (Nameroff and Munar, 1976).

An inhibitory effect was also observed in a dose-dependent manner with ATF (Quax et al., 1992a), stressing the important role of uPAR in muscle cell fusion in vitro. Inhibition of fusion induced by ATF, or by antibodies to uPA or PAI-1, was not associated with inhibition of muscle cell differentiation at the biochemical and molecular levels. Indeed, while muscle cell fusion rates dropped, total CK activity was unchanged or slightly increased, the CPK-MM isoform rate was unchanged, and levels of both myogenin and Id mRNAs were non significantly decreased.

Taken together, these data suggest that the urokinase system is implicated in fusion through mechanisms that do not primarily involve the intrinsic program of muscle cell differentiation. Both uPA and uPAR have been previously localized at the cell-to-cell and cell-to-substratum (focal) contact sites (Pöllänen et al., 1987; Blasi, 1993). It has been stressed that only cells capable of migration express uPAR, and monocyte polarization in response to a chemotactic stimulus recruits both uPA and uPAR at the leading edge of cell migration (Estreicher et al., 1990; Nykjaer et al., 1994; Gyetko et al., 1996; Kindzelskii et al., 1996). Since cell migration is a prerequisite for myoblast fusion in vitro, we assume that modulation of the urokinase system is instrumental in fusion through its connection with the cell migration process.

The receptor-binding region of uPA does not involve the catalytic site of uPA (Apella et al., 1987), and it is currently believed that the major function of uPAR is to retain and concentrate uPA at the cell surface, allowing cell-associated generation of plasmin, pericellular matrix proteolysis, and cell motility (Ellis et al., 1990). In support of this concept, it has been shown that invasion of several neoplastic cells in vitro depend on plasminogen activation mediated by uPA or uPA bound to uPAR (Ossowski and Reich, 1983; Mignatti et al., 1986; Ossowski, 1988; Meissauer et al., 1991). In the present study, exposure to uPA-anticalytic antibodies was associated with inhibition of muscle cell fusion. However, aprotinin failed to inhibit muscle cell fusion, implying that plasmin formation is not necessary for fusion. The inhibition of fusion by anti-uPA antibodies may indicate that the catalytic site of uPA, but not its action on plasminogen, is implicated in cell fusion. This could occur through the uPA/PAI-1 interaction. It may also relate to other proteolytic activities of uPA. For instance, uPA...
induces a direct cleavage of fibronectin (Quigley et al., 1987, 1990; Gold et al., 1992), a molecule known to be implicated in cell motility. Exposure to antibodies to PAI-1, a molecule that binds the catalytic site of uPA and controls uPA catalytic activities (Ellis et al., 1990; Pöllänen et al., 1991; Mimuro et al., 1992), was also associated with an inhibition instead of an activation of muscle cell fusion. This inhibitory effect could be due to altered interactions between uPA and PAI-1, and also between PAI-1 and ECM. Finally, phenomena other than proteolysis are likely implicated in the migratory and fusion process.

To achieve maximal migration, a cell needs appropriate adhesion, i.e., not too strong and not too stable, to the extracellular matrix (ECM) (DiMilla et al., 1993; Lauffenburger and Horwitz, 1996). Interestingly, the uPAR:uPA system mediates cell adhesion independent of its proteolytic function (Wei et al., 1994), and ECM (vitronectin)-attached PAI-1 appears important for assisting migration, in addition to its role in proteolysis control (Seifert et al., 1990; Seebacher et al., 1992). While total uPAR increases in cell lysates prior to cell fusion, the number of receptors free of uPA remains unchanged, which likely indicates that the cell membrane is nearly saturated by endogenous uPA. The binding of the uPAR:uPA complex to PAI-1 results in an uPAR/uPA/PAI-1 complex which is immediately internalized and degraded (Cubells et al., 1990; Conese et al., 1995). In addition, uPAR mediates mechanical force transfer across the muscle cell surface to the cytoskeleton (Wang et al., 1995), which may be important in the cytoskeletal reorganization that occurs at the leading edge of migrating cells (Lauffenburger and Horwitz, 1996). In brief, the urokinase system is involved in both adhesion to the ECM and mechanical force transfer, two crucial events in cell translocation (Wang et al., 1995).

The finding that addition of uPA to a cell culture decreases the fusion rate could be related to competition between exogenous uPA and cell-attached uPA at the level of ECM-attached PAI-1. While total uPAR increases in cell lysates prior to cell fusion, the number of receptors free of uPA remains unchanged, which likely indicates that the cell membrane is nearly saturated by endogenous uPA. Exogenous uPA that could not be captured by already saturated uPAR at the cell surface is able to bind ECM-attached PAI-1, which can impede bridging of the cell membrane to the ECM.

We conclude that: (1) muscle cell fusion largely depends on interactions between the members of the urokinase tripartite complex (uPAR/uPA/PAI-1); (2) the fusion process can be dissociated from the muscle cell differentiation program; (3) muscle cell fusion is a plasmin-independent phenomenon; and (4) the urokinase complex appears involved in muscle cell fusion through its connection with the cell migration process, likely behaving as an adhesion system bridging the cell membrane to the ECM. This, together with the mechanotransducer property of uPAR (Wang et al., 1995), delineates a non conventional role for urokinase, in addition to plasminogen activation.

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