Antibody-mediated Targeting of the Urokinase-type Plasminogen Activator Proteolytic Function Neutralizes Fibrinolysis in Vivo*

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Urokinase-type plasminogen activator (uPA) plays a central role in tissue remodeling processes. Most of our understanding of the role of uPA in vivo is derived from studies using gene-targeted uPA-deficient mice. To enable in vivo studies on the specific interference with uPA function in mouse models, we have now developed murine monoclonal antibodies (mAbs) directed against murine uPA by immunization of uPA-deficient mice with the recombinant protein. Guided by enzyme-linked immunosorbent assay, Western blotting, surface plasmon resonance, and enzyme kinetic analyses, we have selected two highly potent and inhibitory anti-uPA mAbs (mU1 and mU3). Both mAbs recognize epitopes located on the B-chain of uPA that encompasses the catalytic site. In enzyme activity assays in vitro, mU1 blocked uPA-catalyzed plasminogen activation as well as plasmin-mediated pro-uPA activation, whereas mU3 only was directed against the first of these reactions. We additionally provide evidence that mU1, but not mU3, successfully targets uPA-dependent processes in vivo. Hence, systemic administration of mU1 (i) rescued mice treated with a uPA-activatable anthrax proteotoxin and (ii) impaired uPA-mediated hepatic fibrinolysis in tissue-type plasminogen activator (tPA)-deficient mice, resulting in a phenotype mimicking that of uPA−/−PA double deficient mice. Importantly, this is the first report demonstrating specific antagonist-directed targeting of mouse uPA at the enzyme activity level in a normal physiological process in vivo.

Tissue remodeling and cell migration are dependent on the confined degradation of proteins in the extracellular matrix and basement membranes, which is accomplished by the concerted action of several proteolytic enzymes, including those of the plasminogen activation and the matrix metalloproteinase systems (1, 2). The two main proteases accomplishing plasminogen activation, i.e. conversion of plasminogen to the active broad spectrum serine protease plasmin, are urokinase-type plasminogen activator (uPA) 2 and tissue-type plasminogen activator (tPA). At the molecular level, uPA is secreted as an inactive single-chain protein, pro-uPA, which becomes activated by proteolytic cleavage believed to be primarily performed by plasmin (3, 4). The simultaneously occurring reciprocal zymogen activation reactions, composed of pro-uPA activation by plasmin and plasminogen activation by uPA, constitute a positive feedback mechanism, which is regulated at several levels. The naturally occurring inhibitors include serpins and in particular the plasmin activator inhibitor-1 (PAI-1) for uPA and α 2 -antiplasmin for plasmin (1). Although the plasminogen activation processes can be focused to the cell surface by the uPA receptor (uPAR) and plasminogen-binding proteins (5), additional functions of uPA seem to rely on a multifaceted array of interactions with cellular receptors, adhesion molecules, and extracellular matrix proteins (6). A key role for uPA-mediated plasminogen activation has been suggested in tissue remodeling processes and decisively demonstrated by studies of skin wound healing (7–9), embryo implantation (10), and mammary gland involution (11) in gene-targeted knockout mice.

The primary functions of the counterpart of uPA, tPA, have traditionally been associated with intravascular fibrinolysis through a preferential induction of plasminogen activation upon simultaneous association of tPA and plasminogen with fibrin (12, 13). However, despite the apparent differences in their basic biological functions, functional redundancy between uPA and tPA has been demonstrated in various physiological settings using gene-deficient mice. Hence abolishment of single components of the plasminogen activation system, i.e. uPA (13), tPA (13), and uPAR (14, 15), by gene targeting does not affect the viability of the homozygous knock-out animals as they by and large exhibit normal phenotypes with respect to growth, reproduction, and survival. In contrast, mice with combined deficiency in uPA and tPA are characterized by retarded growth, reduced fertility, and shortened life span (13). Moreover whereas a modest but definite delay in incisional skin

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2 The abbreviations used are: uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; uPAR, uPA receptor; mAb, monoclonal antibody; ATF, amino-terminal fragment of uPA; LMW-uPA, low molecular weight uPA; PrAg-U2, uPA-activable anthrax toxin-protective antigen-U2; FP59, fusion protein 59; PBS, phosphate-buffered saline; TNP, trinitrophenyl; SPR, surface plasmon resonance; ELISA, enzyme-linked immunosorbent assay; MSV, mouse sarcoma virus.

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wound healing is observed in mice with single deficiency in uPA, the concomitant ablation of both of the plasminogen activator genes significantly retarded the healing process (7, 8). Detailed studies on hepatic fibrinolysis in gene-deficient mice have in addition revealed extensive fibrin accumulations in uPA;tpA and uPAR;tpA double deficient mice with characteristics resembling those observed in plasminogen-deficient mice (8, 13, 16). Among the single deficient mice, only the uPA knock-out mice developed detectable hepatic fibrin plaques and only occasionally (8, 13). These results strongly argue that a complex pattern of redundancies exists between uPA and tPA. Consequently increasing attention has been drawn to the physiological impact of uPA-induced effects mediated independently of both tPA and of its receptor, uPAR (8, 17, 18).

Taken together, the multitude of protease interaction pathways and the potential redundancy both between the plasminogen activators and with other extracellular proteases (such as the matrix metalloproteinases) require further investigations of the roles of uPA in mouse models. A number of pathological conditions, including cancer invasion and metastasis, have mechanistic similarities to normal physiological tissue remodeling processes, and several lines of evidence support the hypothesis that plasminogen activation mediated by uPA is a key regulatory step in these proteolytic events (1, 6). As the majority of the in vivo investigations concerning uPA have used antagonists directed against the human form of uPA without cross-reactivity to murine uPA (for a review, see Ref. 19), there is thus a strong need for inhibitors of mouse uPA with applicability in vivo. To be able to investigate uPA-mediated effects in diverse physiological settings, we have now generated murine inhibitory monoclonal antibodies (mAbs) against mouse uPA. Such murine uPA antagonists allow for analysis of the in vivo effects on uPA functionality in a range of murine models.

In the present study, we performed a detailed biochemical and functional characterization of two murine anti-mouse uPA mAbs possessing epitopes located on the B-chain of uPA and having different mechanisms of action on uPA functionality. The in vivo efficacy of each antibody was tested by systemic administration to mice treated with a modified anthrax protease, requiring uPA-mediated activation, and the most potent mAb was further used for investigation of the physiological effect of acute disruption of uPA activity in adult mice.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Proteins**

Recombinant proteins were produced by use of a Drosophila Expression System (Invitrogen). The construction of appropriate expression vectors, subsequent stable transfection into Drosophila S2 cells, and large scale production and purification have been described in detail previously (20, 21) for the listed proteins: murine pro-uPA (residues 1–413), human pro-uPA (residues 1–411), the murine amino-terminal fragment of uPA (ATF) (residues 1–143), and soluble murine uPAR (residues 1–275). Two preparations containing either (i) active murine uPA alone or (ii) murine pro-uPA/uPA together with the corresponding proteolytic cleavage products, i.e. ATF and low molecular weight (LMW)-uPA, were produced by purification of medium collected from a subline of cultured mouse sarcoma virus-infected 3T3 cells (i.e. 3T3/MSV-LO) as described previously (22).

**Miscellaneous Reagents**

The following proteins were purchased from the commercial sources indicated: human Glu-plasminogen and plasmin (Kordia Life Sciences, Leiden, the Netherlands) and murine tPA, PAI-1, two-chain uPA, and LMW-uPA (Molecular Innovations, Novi, MI). The chromogenic substrates S2251 and S2444 were purchased from Chromogenix (Hemochrom Diagnostica AB, Mölndal, Sweden). The uPA-activable anthrax toxin-protective antigen-U2 (PrAg-U2) and the cystotoxic fusion protein 59 (FP59), consisting of the anthrax toxin lethal factor residues 1–254 fused to the ADP-ribosylation domain of Pseudomonas exotoxin A, were kind gifts from Dr. S. Liu and Dr. S.H. Leppla, NIAID, National Institutes of Health, Bethesda, MD.

**Mice and Mouse Breeding**

Breeding of mice of different genotypes and strains (specified below under “Experimental Procedures”), including backcrossing to the indicated background and interbreeding to obtain single and double deficient mice as well as wild-type littermates, were performed as described previously (8, 13, 14). Mice were bred at the Panum Institute, Copenhagen, Denmark. The genotypes were identified by PCR of genomic tail DNA (7). The animal experiments in this study were conducted according to institutional guidelines and were approved by the Danish Animal Experiments Inspectorate (2007/561-1353). All mice were found to be free of specified murine pathogens in accordance with the Federation of European Laboratory Animal Science Associations recommendations for health monitoring of experimental units. Mice used for experiments were between 6 and 7 weeks old at the start of the experiment. Experimental evaluation, tissue isolation, and microscopic analyses were performed by investigators unaware of the mouse genotype and treatment.

**Generation of Murine Monoclonal Antibodies against Murine uPA**

Female FVB/n mice deficient in uPA (13) were used for immunization. Mice were injected intraperitoneally at least six times with 2-week intervals followed by 3 days of daily boosts just prior to the day of the cell fusion. 25 μg of recombinant mouse pro-uPA was administered in each injection either diluted in 0.3 ml of isotonic NaCl with 2 mg/ml Al(OH)₃ for standard immunizations or in 0.3 ml of phosphate-buffered saline (PBS), pH 7.2, for boosting injections. The techniques for isolation of peritoneal macrophages from non-immunized mice and preparation of splenocytes from the immunized mice as well as the subsequent fusion between splenocytes and X63-Ag8.653 murine myeloma cells were performed as described previously (21), except that in the present study we used uPA-deficient FVB/n mice. Hybridomas producing antibodies specific for mouse uPA were cloned by limited dilution. The subtypes of the mAbs were determined by the mouse mAb isotype kit (AbD Serotec, Düsseldorf, Germany). Production and purification of mAbs obtained from the hybridoma culture medium were done as described previously (21), except that large scale
production of mAbs for in vivo experiments was performed using Cellline AD 1000 Bioreactors (Integra Bioscience), in which cells were suspended in culture medium (21) additionally supplemented with 10% glucose and 10% (v/v) ultralow IgG fetal calf serum. The large quantities of mU1, mU3, and the isotype-matched monoclonal anti-trinitrophenyl (anti-TNP) antibody (21) were routinely analyzed for endotoxin levels, using the Limulus Amebocyte QCL-1000 lysate method according to the manufacturer’s instructions (BioWhittaker, Walkersville, MD), and found to be endotoxin-negative (i.e. level below 0.4 endotoxin units/g of mouse body weight/injection).

**ELISA Screening**

The initial screening for hybridomas producing antibodies against mouse uPA was performed by ELISA essentially as described previously (21), except that the microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 2 ng/well purified recombinant mouse pro-uPA diluted in coating buffer (0.1 M Na₂CO₃, pH 9.8). A preparation of eye blood serum (diluted 1:2000 in assay buffer, i.e. 50 mM sodium/potassium phosphate, pH 7.4, 0.1 M NaCl, 1% (w/v) bovine serum albumin, and 0.1% (v/v) Tween 20) from two immunized mice was used as positive control; uncoated plates were included as a control for unspecific binding. Absorbance was measured at 490 nm in a SpectraMax Plus384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) with background subtraction (absorbance at 540 nm recorded simultaneously).

**Surface Plasmon Resonance (SPR) Analysis**

SPR analyses on a BIAcore 2000™ instrument (BIAcore, Uppsala, Sweden) were used to (i) perform a secondary screening of the ELISA-positive samples for hybridomas secreting antibodies recognizing uPA, (ii) analyze the gross location of binding sites of the purified anti-uPA mAbs on mouse uPA, and (iii) measure the binding and dissociation rate constants for determination of the equilibrium dissociation constants (K_d) of the uPA-antibody interactions. For these experiments, the indicated proteins were dissolved in coupling buffer (10 mM sodium acetate, pH 5.0) and immobilized on CM5-type sensor chips by amine coupling according to the manufacturer’s instructions. Screening of hybridomas was performed by injection of conditioned medium from which cell debris had been removed by a 15-min centrifugation at 20,000 × g. Binding and dissociation were followed essentially as described previously (21) except that the immobilized proteins were mouse and human pro-uPA and a rabbit anti-mouse Fcγ antibody (BIAcore). Analyses of uPA binding sites and mutual exclusion of binding on uPA of the purified mAbs (see “Results”) were performed by immobilization of uPAR or the mAb in question on the chip and injection of 100 nM pro-uPA followed by an injection of an equimolar amount of uPAR or each of the anti-uPA mAbs. For affinity determinations, each mAb was immobilized at a low density (mU1, 406–2281 resonance units; mU3, 500–1582 resonance units), and a 2-fold dilution series of murine pro-uPA or two-chain uPA was injected (0.4–100 nM) at a flow rate of 50 µl/min at 20 °C. Sensorgrams, obtained from a parallel mock-coupled flow cell as well as appropriate buffer runs, served as blanks allowing subtraction of the bulk. Data were analyzed using the BIAevaluation software, version 3.2 RC1 (BIAcore). The association and dissociation rate constants were calculated by local fitting of the obtained binding curves to a 1:1 Langmuir binding model to allow determination of the K_d of the interactions.

**Western Blot Analysis**

The antigens used in Western blotting were human pro-uPA and the murine proteins pro-uPA, two-chain uPA, LMW-uPA, ATF, tPA, and uPA-PAI-1 complexes (prepared by mixing uPA with a 30-fold excess of PAI-1 overnight at 4 °C) as well as the partially degraded uPA preparation containing pro-uPA/uPA, ATF, and LMW-uPA (described above). Electrophoretic separation by SDS-PAGE was performed under non-reducing conditions when not stated otherwise, and Western blotting was performed as described previously (21), except that the membranes were probed with undiluted hybridoma medium or 2 µg/ml purified anti-uPA mAb. When reduced and alkylated uPA was probed, 20 µg/ml purified mAb was used. As a positive control, a preparation of eye blood serum from two immunized mice, diluted 1:30,000 in assay buffer (Tris-buffered saline containing 2% (w/v) skimmed milk powder), was applied.

**Cell Binding Experiments**

Measurement of the uPA-uPAR interaction on the surface of murine monocyte macrophage-like cells (P388D.1, ATCC TIB-63) was performed as described previously (21) with slight modifications: 0.1 nM 125I-pro-uPA was mixed with either 100 nM unlabeled pro-uPA, mU1, mU3, anti-TNP, or PBS alone and allowed to incubate for 45 min at 4 °C before being added to the cells for a further 1-h incubation at 4 °C. The reported standard deviation values are calculated from at least three independent experiments.

**Measurements of Plasminogen and Pro-uPA Activation**

Three different experimental settings were used to evaluate mAb-induced effects on mouse uPA: (i) uPA-mediated plasminogen activation, (ii) plasmin-mediated pro-uPA activation, and (iii) coupled pro-uPA and plasminogen activation. The kinetic assays were performed and presented accordingly to Behrendt et al. (40) except for the changes specified below. The components indicated below were mixed in assay buffer (0.1 M Tris/ HCl, pH 7.4, with 0.1% (v/v) Tween 80; final volume of 100 µl/well). The reactions, performed at 37 °C, were initiated by addition of substrate and plasminogen (as the final components (t = 0 min), and substrate conversion was followed by recording the absorbance at 405 nm for up to 30 min in a SpectraMax Plus384 microplate spectrophotometer. Each curve depicts percent activity with 100% representing the activity of the protease (plasmin or uPA) in the absence of inhibitor at the assay end point. As negative controls, samples prepared by excluding (pro)-uPA, plasminogen, or substrate were run in parallel and showed no activity. Data were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

**uPA-mediated Plasminogen Activation**—0.6 nM mouse uPA, 100 nM human Glu-plasminogen, and 0.8 nM chromogenic plasmin substrate H-d-Val-Leu-Lys-p-nitroanilide (S2251)

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were incubated in the absence or presence of the indicated concentrations of the antibodies (mU1, mU3, and anti-TNP) or other inhibitors as indicated.

**Plasmin-mediated Pro-uPA Activation—**1.6 nM mouse pro-uPA, 0.5 nM human plasmin, and 0.8 nM chromogenic uPA substrate pyro-Glu-Gly-Arg-p-nitroanilide (S2444) were incubated in the absence or presence of 0.03–67 nM mU1, 2–333 nM mU3, 67 nM anti-TNP, or other inhibitors as indicated. The same substrate was used in a variant of this assay analyzing the effect of the antibodies against the amidolytic activity of preactivated uPA. These measurements were performed by mixing 0.5 nM mouse two-chain uPA with 0.8 mM S2444 in the presence or absence of 10 nM mU1, mU3, or PAI-1.

**Coupled Pro-uPA and Plasminogen Activation—**0.5 nM mouse pro-uPA, 20 nM human Glu-plasminogen, and 0.8 mM S2251 were incubated in the absence or presence of the indicated concentrations of mU1, mU3, or anti-TNP. The uPA inhibitors PAI-1 (0.03–67 nM) and amiloride (0.20–200 μM) as well as the plasmin inhibitors α2-antiplasmin (1–67 nM) and aprotinin (2–134 nM) were included as controls in the assays where indicated.

**Half-life Determinations of the Anti-uPA mAbs in Wild-type Mice**

Female C57Bl/6j mice (n = 6) received either 20 mg/kg mU1 or 60 mg/kg mU3, diluted in PBS, by intraperitoneal injections. Citrate plasma samples were made from tail vein blood, which was taken before dosing as well as 8–120 h after injections of the mAbs. The plasma concentrations of the mAbs were analyzed by ELISA as described above, except that coating was increased to 4 ng of mouse pro-uPA/well. The half-lives of the mAbs were calculated from the logarithmic value of the antibody concentrations during the linear elimination phase.

**In Vitro and in Vivo uPA-activable Anthrax Toxin Experiments**

Cytotoxicity assays in vitro were performed using the murine monocytic macrophage-like P388D.1 cells and were carried out as described previously (21) with the following modifications: after acid wash of the cells, 2.2 nM recombinant mouse pro-uPA and 11.1 nM human Glu-plasminogen in serum-free medium in the absence or presence of a 2–1067 nM concentration of the specified mAbs or 1–630 nM recombinant mouse ATF were added, and cells were preincubated for 0.5 h at 37 °C. PrAg-U2 and the cytotoxic FP59 component (23) were subsequently added to give final concentrations of 75 and 12.5 ng/ml, respectively. After an additional 3 h of incubation, the medium was replaced with fresh serum-supplemented culture medium, and at 48 h cell viability was measured by use of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega, Madison, WI) as described previously (21). Absorbance was measured at 570 nm in a SpectraMax Plus384 microplate spectrophotometer with appropriate corrections for absorbance of non-enzymatic hydrolysis of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide substrate. Results are given as percentage of control, which is the viability of cells incubated in parallel but in the absence of ATF, mAbs, and the anthrax toxin mixture. The reported standard deviation values are calculated from at least four independently performed experiments.

To examine the in vivo efficacy of mU1 and mU3, experiments using the uPA-activable anthrax protoxin were performed essentially as described previously (21), except that only female FVB/n wild-type mice were used and that female uPA gene-deficient mice (13) of the same strain were included as a control group. The mice were given intraperitoneal injections of the indicated antibody (60 mg/kg) or an equivalent volume of PBS alone at days −1 and 0 followed by administration of the anthrax toxin (0.6 mg/kg PrAg-U2 + 0.4 mg/kg FP59) at day 0. The mice were observed for signs of toxicity twice a day, and weight and death events were recorded for a total period of 7 days. Data from cell and animal experiments were analyzed by GraphPad Prism 4 software (GraphPad Software), and survival of the mice was presented as Kaplan-Meier curves.

**Detection and Quantification of Hepatic Fibrin Clots**

Female wild-type and tPA-deficient FVB/n mice (9–10 mice per group) were injected intraperitoneally with mU1 diluted in PBS or with the equivalent volume of PBS alone. The treatment was initiated with a loading dose of 100 mg/kg, and antibody plasma levels were maintained by administration of 50 mg/kg mU1 twice weekly for 6 weeks. Preparation, fixation, and staining of the hepatic tissue, isolated from the mice for histological and immunofluorescence examination as well as subsequent quantification of fibrin immunofluorescence signal, were performed as described previously (24), except that a polyclonal rabbit anti-mouse fibrinogen antibody (1:2000 dilution) and an Alexa Fluor 488 goat anti-rabbit antibody (1:200 dilution, catalog number A-11070, Invitrogen) were used as primary and secondary antibodies, respectively. For comparison, liver sections were taken from untreated wild-type, tPA-deficient, uPA-deficient, and uPAtPA double deficient FVB/n mice, respectively, and analyzed in the same manner. The statistical analyses, comparing the percentage of the liver area with fibrin immunofluorescence signal of all treated groups of mice, were made using one-way analysis of variance using the SAS System, version 6.12 (SAS Institute, Cary, NC). Values were considered statistically significant at p < 0.05.

**RESULTS**

**Generation of Murine Monoclonal Antibodies Targeting Murine uPA—**uPA-deficient mice were immunized with recombinant mouse uPA followed by fusion of splenocytes isolated from the immunized mouse with myeloma cells using the conventional hybridoma technique (25). Guided by ELISA, SPR analysis, and Western blotting, nine hybridomas, which secreted antibodies reacting with uPA, were selected from two independently performed fusions and cloned by limited dilution. We decided on two of these monoclonal-produced antibodies for the further characterization as they demonstrated high specificity and selectivity for mouse uPA in Western blotting using culture medium collected from murine 3T3/MSV-LO cells as antigen (results not shown). These mAbs, both of the IgG1 isotype, were designated mU1 and mU3. Neither of the mAbs recognized the human form of pro-uPA, thus defining their epitopes on murine uPA as composed of non-conserved amino acids, nor did they cross-react with...
Eptope Location of the mAbs on Mouse uPA—uPA is secreted from cells as an inactive, single-chain proenzyme, pro-uPA. Upon plasmin-induced activation of mouse pro-uPA, the primary proteolytic cleavage occurs at Lys<sup>136</sup>-Ile<sup>139</sup> (numbering according to the murine sequence) (26), generating the active two-chain uPA enzyme. Further proteolytic processing of murine uPA at Lys<sup>136</sup>-Lys<sup>137</sup> produces ATF (residues 1–136) and LMW-uPA (residues 137–413) (26) of which the latter encompasses the serine protease domain linked by a disulfide bridge to part of the connecting peptide. As special functions can be assigned to each of the structural domains of uPA, the epitope locations of the mAbs are likely to be indicative of their effects on uPA functionality. Application of various murine uPA preparations including recombinant full-length pro-uPA (amino acids 1–413; Fig. 1A, lane 1), ATF (the recombinant form comprising amino acids 1–143; lane 2), and a purified solution containing 3T3/MSV-LO cell-produced pro-uPA/uPA and its corresponding proteolytic cleavage products, i.e. ATF and LMW-uPA (lane 3), revealed the epitopes of mU1 and mU3 to be located exclusively on LMW-uPA. Within this fragment, further localization of the binding sites was done by Western blotting of reduced samples, separating the connecting peptide (residues 137–159) from the B-chain (residues 160–413). This experiment revealed that both mAbs were reactive with the B-chain and not the connecting peptide (Fig. 1B), although as expected, the signals obtained were weaker than those found using unreduced samples. Finally both antibodies recognized preformed uPA/PAI-1 complexes in Western blotting (results not shown), hence demonstrating non-overlapping binding sites with that of PAI-1, which is known to bind to the uPA catalytic site (27).

Next by using SPR analysis, we showed that mU1 and mU3 mutually prevented the binding of each other to uPA (supplemental Fig. S1). Thus, although their binding sites are unlikely to be identical (see the functional properties below), they are structurally overlapping in the sense that mutual steric hindrance is complete. Finally these SPR experiments demonstrated that neither of the mAbs interfered with the uPA-uPAR interaction as they were both able to bind to preformed complexes of uPA and uPAR (supplemental Fig. S1). This is consistent with the receptor-binding epitope being located on the ATF part of uPA (28). The latter finding was confirmed in a cellular environment as neither mU1 nor mU3 blocked the binding of <sup>125</sup>I-pro-uPA to uPAR on the surface of murine monocyte macrophage-like P388D.1 cells (Fig. 1C). On the contrary, an apparent increase in binding of <sup>125</sup>I-pro-uPA was noted in the presence of both antibodies, most likely resulting from divalent antigen binding and avidity effects.

To obtain a quantitative estimation of the affinities of mU1 and mU3 for pro-uPA and two-chain uPA, we examined their binding kinetics using SPR analysis. Both antibodies were found to possess high affinities for pro-uPA and two-chain uPA, i.e. equilibrium dissociation constants (K<sub>D</sub>) in the low nanomolar range (Table 1). Notably with both pro-uPA and active uPA, mU3 possessed a severalfold higher affinity than mU1.

mAb-induced Inhibition of Plasminogen Activation—Using an enzyme kinetic assay designed to measure the conversion of a chromogenic plasmin substrate, we first investigated the effect of mU1 and mU3 on uPA-mediated plasminogen activation. As illustrated in Fig. 2, A and B, both antibodies inhibited uPA-catalyzed plasminogen activation in a dose-dependent manner. mU1 displayed half-maximal inhibition at a concentration of approximately 2 nM, whereas mU3 was even more efficient with half-maximal inhibition between 0.1 and 0.5 nM. The reaction was unaffected by addition of the isotype-matched control antibody, anti-TNP (data not shown). Comparison with naturally occurring inhibitors showed that, as expected, a dose-dependent inhibition of uPA could be obtained in the same assay with PAI-1 (Fig. 2C), whereas the...
TABLE 1
Kinetic parameters for the binding of the anti-uPA mAbs to pro-uPA and two-chain uPA

|        | Pro-uPA | Two-chain uPA |
|--------|---------|--------------|
|        | $k_a$   | $k_d$ | $K_D$ | $k_a$   | $k_d$ | $K_D$ |
| mU1    | $(1.5 \pm 0.4) \times 10^5$ | $(2.8 \pm 0.5) \times 10^{-4}$ | 0.2 | $(6.4 \pm 2.1) \times 10^5$ | $(8.3 \pm 1.3) \times 10^{-4}$ | 1.3 |
| mU3    | $(7.7 \pm 3.5) \times 10^5$ | $(2.6 \pm 1.2) \times 10^{-4}$ | 0.03 | $(2.1 \pm 1.1) \times 10^6$ | $(4.0 \pm 2.1) \times 10^{-4}$ | 0.2 |

*Note that the preparations of pro-uPA and active uPA were derived from different cellular sources and cannot necessarily be compared with respect to mAb affinity; see Experimental Procedures.*

generated plasmin activity could be completely quenched by either of the two plasmin inhibitors, α$_2$-antiplasmin (5 nM) and aprotinin (67 nM) (results not shown). With amiloride, a low molecular weight uPA inhibitor, a concentration of 200 μM was found to be required to block uPA activity under the same conditions (results not shown).

As activation of pro-uPA is an initial step in the plasminogen activation system, an antagonist of this reaction would also be a highly attractive candidate for complete inhibition of the cascade. Therefore, in a parallel experiment, we monitored the effect of the mAbs against plasmin-mediated pro-uPA activation using a chromogenic uPA substrate. In this setup, mU1 turned out to be capable of strongly inhibiting the activation of pro-uPA even in a concentration as low as 0.5 nM (Fig. 2D). Importantly mU3 in contrast had no effect in this latter assay. As expected, comparison with other inhibitors, as above, showed that dose-dependent inhibition could be obtained with both PAI-1 and amiloride directed against the activity of the generated two-chain uPA, whereas a complete abrogation was obtained with molar excess of α$_2$-antiplasmin and aprotinin relative to plasmin (results not shown).

In a third version, we used a coupled assay with pro-uPA, plasminogen, and the plasmin substrate, including both of the two activation reactions (40). In this setup, both mAbs were capable of inhibiting the composite reaction efficiently and with roughly the same concentration dependence (Fig. 2E). Thus, under these conditions, the fact that mU1 was efficient against both activation reactions apparently compensated for its lower activity against two-chain uPA relative to mU3. In the same assay, equimolar concentrations of α$_2$-antiplasmin and aprotinin completely inhibited the generated plasmin activity (results not shown), whereas abrogation of the resulting uPA activity, as expected, required an excess of PAI-1 and amiloride (Fig. 2F).

Finally we wanted to learn whether a direct blocking of the catalytic site of uPA was involved in the inhibition phenomena. For this purpose, we measured the effect of the mAbs against the amidolytic activity of two-chain uPA with the uPA substrate (S2444; supplemental Fig. S2). It was evident that mU3 had no effect on this activity, whereas any effect of mU1 was only mar-
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Original. Altogether in the integrated system the effect of mU1 seems to include both steric hindrance, leading to a blocked interaction with plasminogen at the active site, and decreased plasmin-mediated pro-uPA activation, whereas the effect of mU3 is only directed against the uPA-mediated plasminogen activation.

mU1 Rescues Mice Treated with uPA-activable Anthrax Protoxin—The unique modified anthrax toxin assay in which cytotoxicity is dependent on a specific cleavage of an engineered protective antigen, i.e. PrAg-U2, catalyzed by uPAR-bound uPA (23) was used to assess the uPA-targeting effects of mU1 and mU3 in vitro and in vivo. Antibody-induced effects on uPA functionality regarding its activation, catalytic activity, and interaction with uPAR could thus be evaluated in a multicomponent cellular system. In the absence of antibody or other uPA-blocking reagents, exposure of murine monocyte macrophage-like P388D.1 cells to PrAg-U2 and the recombinant cytotoxin FP59, which upon binding to uPA-activated PrAg-U2 becomes internalized, leads to toxicity-caused cell death. Based on the biochemical characteristics of mU1 and mU3, we speculated that these mAbs could block the critical function of uPA in this cellular system. Indeed a significant rescue of cells treated with the uPA-activable anthrax protoxin was obtained upon incubation of the cells with either of the antibodies (Fig. 3A). The dose-dependent inhibition of uPA-directed functions induced by mU1 almost reached the level of the positive control, mouse ATF, which possesses no proteolytic potential and acts to compete with uPA for binding to uPAR on the target cells. Thus, this experiment demonstrated a strong inhibitory impact of mU1 on uPA catalytic activity in this multicomponent system in vitro, whereas the rescue obtained with mU3 was slightly less pronounced.

Previous reports have demonstrated uPA gene-deficient mice to be insensitive to treatment with high doses of PrAg-U2 + FP59 (29), highlighting uPA activity as an absolute requirement for activation of PrAg-U2 and internalization of FP59 in vivo. To assess the in vivo efficacy of mU1 and mU3, we therefore investigated whether administration of the mAb in question, prior to treatment with an otherwise lethal dose of PrAg-U2 + FP59, could attenuate the toxicity induced by this toxin in wild-type mice. This study was preceded by an experiment with administration of mU1 and mU3 to wild-type mice without toxin treatment where the half-lives of the two mAbs in circulation were determined to be ~3 and 6 days, respectively (data not shown). Accordingly because severe toxicity of the PrAg-U2 + FP59 mixture, ultimately resulting in death, occurs within 3–4 days, two intraperitoneal consecutive injections containing either mU1 or the control mAb anti-TNP (both at 60 mg/kg) were administered to the mice before the anthrax toxin injection (0.6 mg/kg PrAg-U2 + 0.4 mg/kg FP59) was initiated. Notably 80% (eight of 10 mice) of the wild-type mice treated systemically with mU1 followed by an anthrax toxin injection survived, whereas only 30% (three of 10 mice) of each of the two groups receiving either toxin alone or toxin and anti-TNP survived (Fig. 3B and results not shown). A separate study conducted to test the effect of mU3 and including mU1 as control revealed that no considerable rescuing effect was achieved upon administration of mU3 to the toxin-treated mice (seven of 10 mice died; supplemental Fig. S3). A group of uPA-deficient mice received anthrax toxin injections without antibody treatment to ascertain the uPA dependence of the experiment. All of these mice were unaffected by the treatment (data not shown) consistent with previous reports (29). Furthermore we included additional control groups, i.e. wild-type mice only receiving either mU1 or mU3. These mice all survived (Fig. 3B and supplemental Fig. S3), indicating that administration of mU1 and mU3 did not cause any lethal or otherwise obvious side effects. These data clearly demonstrate that mU1 in vivo can attenuate the toxicity induced by the uPA activity-dependent anthrax toxin by impeding activation of PrAg-U2 and subsequent internalization of FP59.

mU1 Treatment Induces Accumulation of Hepatic Fibrin in tPA-deficient Mice—Combined gene targeting of uPA and tPA results in spontaneous development of considerable fibrin accumulations in the liver, and occasionally this is also observed in uPA single deficient mice (8, 13). To further explore
Specific Inhibition of uPA Activity in Vivo

The objective of the present study was to analyze the effect of specifically targeting the proteolytic activity of uPA in live mice. This was achieved using murine monoclonal antibodies directed against murine uPA and capable of inhibiting uPA-mediated plasminogen activation without interfering with uPA receptor binding. One monoclonal antibody, mU1, had a particularly strong inhibitory impact on uPA functionality both in vitro and in vivo, an effect that most likely comprises different steps of the plasminogen activation cascade, i.e. uPA-catalyzed plasminogen activation and plasmin-induced activation of the uPA proenzyme.

The biological consequence of mU1 on a uPA activity-dependent process in vivo, we examined the mU1-induced effect on hepatic fibrin clearance by systemic treatment of tPA-deficient mice. First in a separate experiment, we analyzed the prevalence of hepatic fibrin accumulations in untreated FVB/n mice, including all four genotypes, i.e. wild-type, uPA and tPA single deficient, and uPA;tPA double deficient mice. In the livers of untreated FVB/n uPA;tPA double deficient mice, we found extensive fibrin deposits (Fig. 4A) as reported previously for the equivalent double deficient mice in the C57Bl/6J background (8). Compared with the rare and tiny fibrin deposits that were detectable in wild-type and tPA and uPA single deficient mice, the frequency and size of fibrin plaques in uPA;tPA double deficient mice were markedly increased (Fig. 4A). Next to determine whether it was possible to reduce the fibrinolytic activity through inhibition of uPA functions by mU1 administration, mice were treated with mU1 or PBS. To achieve steady-state plasminogen levels of the antibody, i.e. in compliance with the 3-day half-life, a loading dose of 100 mg/kg mU1 followed by two weekly injections of 50 mg/kg mU1 was administered to wild-type and tPA-deficient mice throughout the 6-week experiment. An equivalent volume of PBS was given to the control mice. With the exception of the liver, no overt effects induced by mU1 were observed in any organs, locally at the site of administration, or on weight gain of the mice (results not shown). Strikingly systemic administration of mU1 resulted in a severalfold increase in hepatic fibrin accumulations in tPA-deficient mice compared with placebo-treated littermates as judged by quantification of the percentage of liver area displaying fibrin immunofluorescence signal (Fig. 4B). The multiplication in fibrin plaques between tPA-deficient mice treated with mU1 or PBS was statistically significant (Fig. 4B; p = 0.002, one-way analysis of variance). The level of fibrin plaques observed in the mU1-treated tPA-deficient mice was only slightly lower than and not statistically different from the level observed in placebo-treated uPA;tPA double deficient mice (Fig. 4C; p = 0.13, one-way analysis of variance).

Notably wild-type mice treated with mU1 did not show any significant increase in hepatic fibrin accumulation compared with placebo-treated wild-type mice or tPA single deficient mice. This latter observation clearly documents that the increased fibrin accumulations in the livers of mU1-treated tPA-deficient mice were caused by a specific blocking of uPA-dependent plasminogen activation and not a result of unspecific reactions unrelated to plasminogen activator activity. In accordance with this notion, previous studies have specifically addressed the putative occurrence of unspecific antibody effects in a similar system. Thus, treatment of wild-type as well as tPA-deficient mice with an irrelevant antibody, anti-TNP, had no effect on hepatic fibrinolysis (24). At the same time, these data underscore the functionally overlapping role of uPA and tPA, which makes specific uPA targeting tolerable in a wild-type animal.

DISCUSSION

In vivo the objective of the present study was to analyze the effect of specifically targeting the proteolytic activity of uPA in live mice. This was achieved using murine monoclonal antibodies directed against murine uPA and capable of inhibiting uPA-mediated plasminogen activation without interfering with uPA receptor binding. One monoclonal antibody, mU1, had a particularly strong inhibitory impact on uPA functionality both in vitro and in vivo, an effect that most likely comprises different steps of the plasminogen activation cascade, i.e. uPA-catalyzed plasminogen activation and plasmin-induced activation of the uPA proenzyme.
Specific Inhibition of uPA Activity in Vivo

Although a large number of research groups over the past decades actively have pursued structure-based design for development of inhibitory compounds against the human form of uPA, only a few reports have described antagonists of murine uPA (for a review, see Ref. 19) despite the fact that the latter are essential for studies in murine models. Furthermore, although antibodies against uPA would be attractive tools to circumvent this problem, the utilization of conventional murine antibodies is typically limited by their lack of reactivity against the murine target proteins, whereas antibodies raised in other species give rise to immunological side reactions (1, 30). Thus, our approach, using immunization of uPA-deficient mice with murine uPA, was chosen to avoid both problems. The versatility of this overall strategy was suggested by a notable study by Declerck et al. (31) on inhibition of mouse tPA that demonstrated the generation of murine anti-tPA mAbs with enzyme-inhibitory function using tPA gene-deficient mice.

The balance in the extracellular milieu between individual proteases and their inhibitors is a critical determinant for the maintenance and plasticity of tissue architecture. In a number of physiological settings, including liver repair after CCl$_4$-induced injury (17), wound healing (7, 8, 18), and hepatic fibrin dissolution (8), genetic deficiency in uPA has been found to cause increased mean healing time and augmented accumulation of fibrin clots, respectively. However, these physiological processes were suggested to rely on uPA-directed functions, possibly being redundant with those of other proteases such as tPA and the matrix metalloproteinases. In consistence, the present study demonstrates that systemic administration of mU1 induces a significant formation of fibrin plaques in the liver sinusoïds specifically in tPA-deficient mice, a physiological effect that mimics one of the phenotypic characteristics of uPA/tPA double deficient mice (8, 13). Hence our results suggest that a monoclonal antibody that directly abrogates uPA catalytic activity is capable of shifting the fibrinolytic balance in adult mice. Similarly we have recently shown that a murine mAb, termed mR1, directed against the mouse uPA receptor and known to prevent uPA-uPAR interaction (21) can be used for systemic treatment of tPA-deficient mice to obtain a phenotype mimicking that of genetically induced uPARtPA double deficient mice (8, 24).

Previously an elegant study by Scott et al. (32) illustrated that mice, either treated with a rat mAb directed against murine tumor necrosis factor-α or rendered tumor necrosis factor-α-deficient by gene targeting, displayed significantly reduced skin tumor development as compared with wild-type littermates. Analogous with our results, the inhibitory mAb was somewhat less efficient than ablation of the target gene (32). The explanation offered by these authors relied on fluctuating levels of the antibody, potentially resulting in residual activity of the target protein. Whereas a similar explanation may indeed lie behind the less than complete effect noted in our work, it is also possible that regulatory mechanisms serve to enhance the efficiency of the uPA system and other proteolytic systems in the tPA-deficient mice, thus making the blocking of uPA activity particularly difficult in these mice. Hence, although valuable information can certainly be obtained through application of different types of gene-targeted models, a number of limitations may occur, masking the functionality of the target protein as a result of counter-regulatory mechanisms, such as up- and down-regulation of other genes and/or increased occurrence of protein(s) possessing redundant functions with the target protein.

Inhibition of extracellular proteases is an attractive approach to cancer therapy (1, 6). An impressive number of studies (for reviews, see Refs. 1, 19, 33, and 34) have in particular focused on uPA as a target and demonstrated that in several types of cancers (i) uPA is overexpressed, (ii) uPA is a strong and independent prognostic marker, and (iii) uPA, together with PAI-1, is a predictive marker for response to adjuvant chemotherapy. Additionally the ablation of uPA functions, either by deletion of the uPA gene in mice or by administration of antagonists against human uPA to xenografted rodents, results in impeded cancer progression (30, 35–38). Hence uPA seems to play a key role during cancer invasion and metastasis, and together with the apparent lack of an adverse phenotype of uPA-deficient mice, this highlights uPA-directed anti-cancer therapy as an attractive experimental approach with limited side effects to be expected. Furthermore evidence demonstrating uPA and its receptor to be expressed by subsets of stromal cells at the invasive front of the cancers has been provided (1). Consequently the application of antagonists such as antibodies against human uPA in xenografted cancer models is limited by their lack of reactivity against mouse uPA derived from the stromal cell components of the tumors and metastases (1).

The general approach of anti-cancer therapy with monoclonal antibodies has been based on antibodies directed against cell surface-anchored proteins or the receptor binding site of a ligand (39). In contrast, only a few studies have described the development of mAbs specifically targeting the catalytic activity of an enzyme. On this basis, it is highly tempting to use mU1 or equivalent murine antagonists for investigations of cancer progression in a number of murine cancer models. The strategy used in this work can probably be further improved by administration of combinations of inhibitors directed against two or several components of the plasminogen and the matrix metalloproteinase systems.

In many respects, the administration of an efficient, immuno-compatible blocking antibody could be considered equivalent to the construction of gene-targeted conditional knock-out mice. However, whereas the latter option has the benefit of creating tissue-specific deficiency of the protein in question, the use of specific mAbs allows for the blocking of just one of several functions of the target protein. Thus, mU1 efficiently blocked the enzymatic activity of uPA without affecting the uPA-uPAR interaction, which is considered essential for the non-proteolytic roles of the uPA system (6). Although various knock-in strategies might be used to achieve a similar goal, these procedures are highly laborious, and importantly antibodies can much more easily be utilized in combination studies in which two or several components are being targeted because genetic techniques require very extensive crossing schemes to obtain homozygous double or triple deficient animals. Thus, the present technique is ideally suited for investigations of the function of the target protein during normal physiological tissue remodeling processes as well as pathological conditions using administration of the antibody alone as well as in combi-
nation with the blocking of other proteases. Furthermore the therapeutic potential of the mAbs is evaluated directly. In this context, it is highly important that the mAb administration in our study caused no obvious side effects in wild-type mice (in which the potential redundant mechanisms are at play).

In conclusion, we have succeeded in generating highly potent murine inhibitors of mouse uPA functionality, and simultaneously we have demonstrated a high degree of in vivo efficacy of one of them in mouse model systems in which uPA plays a critical role. We thus believe that inhibitory murine anti-uPA mAbs can be applied as novel tools to further scrutinize uPA functions in vivo during both normal and pathological tissue remodeling processes.

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