ANTAGONISTIC ANTI-UPAR ANTIBODIES SIGNIFICANTLY INHIBIT UPAR-MEDIATED CELLULAR SIGNALING AND MIGRATION

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Running title: Anti-uPAR antibodies inhibit cellular signaling and migration

Interactions between urokinase plasminogen activator receptor (uPAR) and its various ligands regulate tumor growth, invasion and metastasis. Antibodies that bind specific uPAR epitopes may disrupt these interactions, thereby inhibiting these processes. Using a highly diverse and naïve human fragment of antigen binding (Fab) phage display library, we identified 12 unique human Fabs that bind uPAR. Two of these antibodies compete against urokinase plasminogen activator (uPA) for uPAR binding, while a third competes with β1 integrins for uPAR binding. These competitive antibodies inhibit uPAR-dependent cell signaling and invasion in the non-small cell lung cancer cell line, H1299. Additionally, the integrin-blocking antibody abrogates uPAR/β1 integrin-mediated H1299 cell adhesion to fibronectin (FN) and vitronectin (VN). This antibody and one of the uPAR/uPA antagonist antibodies shows a combined effect in inhibiting cell invasion through Matrigel/Collagen I or Collagen I matrices. Our results indicate that these antagonistic antibodies have potential for the detection and treatment of human uPAR-expressing tumors.

Many of these processes are initiated by the highly specific binding of various ligands to membrane-bound uPAR. One such interaction is between uPAR and uPA, which mediates both extracellular and intracellular signaling events (7-9). Binding of extracellular pro-uPA to uPAR facilitates its activation (10). In turn, uPA activates proteases, such as plasmin, which directly and indirectly degrade the extracellular matrix (ECM). Furthermore, plasmin can activate pro-uPA leading to a positive feedback loop that accelerates ECM degradation.

uPAR is also able to act intracellularly by activating proliferative signal transduction pathways. While many of these proliferative signals are dependent on uPA binding, they are largely independent of uPA’s catalytic activity (11,12). These uPAR-initiated intracellular signaling events are mediated by interaction with other proteins either directly or as part of a multi-protein complex (13). Additionally, uPAR is believed to directly associate with integrin family adhesion receptors in complexes that mediate RGD-independent cell signaling and migration (14). Peptides and small molecules that disrupt uPAR/β1 integrin interactions have been shown to prevent tumor metastasis in animal models (15,16). uPAR’s multi-domain structure enables the binding of diverse ligands (17). In some cases, it has been shown that the presence of uPA increases the affinity between uPAR and its other partners, such as vitronectin (18). Furthermore, uPAR/uPA-dependent signaling seems to require uPAR/integrin interactions (19,20). Thus, uPAR serves to integrate an array of growth and migration signals from the extracellular milieu via...
a network of binding events. Therefore, identifying reagents that block these binding events is an active area of research.

Several peptides, peptidomimetics, small molecules, and antibodies that block uPAR/uPA have been identified (21); however, none of the peptide or small molecule approaches has advanced into clinical studies (22). Recent advances in highly selective antibody therapeutics against extracellular targets have made these molecules attractive reagents for targeting the uPAR/ligand interactions (23); however, fully human antibodies that bind uPAR with high affinity, and interrupt uPA and α5β1 integrin binding, have not been previously described.

Phage display technology provides a facile way to clone large repertoires of human antibody binding regions and screen for molecules that bind to a target such as uPAR. We describe a panel of anti-uPAR antibodies discovered from a highly diverse and naïve human Fab phage display library. Two Fabs that compete with uPA for uPAR binding and one Fab that competes with α5β1 integrin for uPAR binding were identified. These antibodies are capable of selectively labeling uPAR-expressing cells, and inhibit uPAR-mediated cell signaling and migration. In addition, these human anti-uPAR antibodies were used to demonstrate that the inhibition of both the uPAR/uPA and uPAR/α5β1 interactions has a synergistic effect on cellular signaling and cancer cell migration.

**EXPERIMENTAL PROCEDURES**

**uPAR expression and purification**- Human soluble uPAR cDNA (residues 1-277) was ligated into the insect cell expression vector pACgp67 (BD Biosciences). pACgp67 and Baculogold DNA (BD Biosciences) were co-transfected into Spodoptera frugiperda 9 (Sf9) cells using Lipofectamine™ (Invitrogen) and recombinant baculovirus was harvested and amplified according to the manufacturer’s protocol. Sf9 cells were infected with the recombinant baculovirus at an MOI of 0.25, and infected cell culture supernatant was harvested seven days post-transfection.

uPAR was captured by antibody affinity chromatography, eluted, then dialyzed overnight before purification by fast protein liquid chromatography on a MonoQ (GE Life Sciences) column using a linear gradient from 0 to 1 M NaCl for elution.

**Phage Display Library Construction**- A fully human naïve Fab phage display library was constructed using methods described by de Haard et al. (24). Briefly, peripheral blood lymphocyte cDNA was synthesized from RNA. The resulting library was cloned into a phagemid vector, which fuses a C-terminal hexa-histidine and c-myc tag to the heavy chain. Large-scale phage rescue was performed using M13K07 helper phage.

**Phage Display Panning**- Human soluble uPAR was immobilized overnight to a Nunc Maxisorp™ 96-well microplate (eBioScience) at 10 µg/ml in 50 mM sodium carbonate pH 9.5 and unbound uPAR was removed by washing. uPAR-coated wells were then blocked with milk, washed, and a pre-blocked aliquot of the phage library was divided between the wells. Unbound phage were washed away, and bound phage were recovered by adding Escherichia coli (E. coli) TG1 cells. Infected TG1 cells were spread onto selection plates, grown overnight, and harvested by plate scraping. Phage were amplified with M13K07 helper phage infection in liquid culture. Fab-displaying phage were harvested from the culture supernatant and concentrated by PEG precipitation.

The 2nd and 3rd rounds of panning were conducted similarly to the 1st round, but the washing step was made increasingly stringent to remove weakly bound phage.

**Expression of Fab into culture supernatants**. Phage-infected E. coli TG1 colonies were grown in selection media, and Fab expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM final) to cultures showing log phase growth. Cultures were shaken overnight to induce periplasmic Fab expression, a minor portion of which leaks into the culture supernatant. Following overnight incubation, TG1 culture supernatants containing leaked Fabs were collected by centrifugation.

**Preparation of periplasmic fraction**. Cell pellets from phage-infected TG1 cultures grown at the 96-well plate scale and induced for Fab expression by IPTG, were resuspended in 50 µl 100 mM Tris pH 8.0, 25% glucose, 100 µg/ml hen egg white lysozyme and shaken at room
temperature for 30 minutes. 300 µl of ice-cold water was then added and mixed with vigorous pipeting. The periplasmic fraction was then clarified by centrifugation.

**Fab purification** Individual Fab clones were expressed in *E. coli* BL21 cells (as described for TG1 cells). Periplasmic fractions were purified by immobilized nickel chelate chromatography using Chelating Sepharose™ (GE LifeScience) according to the manufacturer’s protocol.

Purified protein was analyzed by SDS-PAGE, and the concentration was estimated with the BCA™ Protein Assay Kit (Pierce), using bovine serum albumin (BSA) standards. Each Fab was analyzed for expression by Western Blot using a Penta-His horseradish peroxidase (HRP) conjugate antibody (Qiagen) according to the manufacturer’s protocol.

**uPAR ELISA.** uPAR-binding Fabs were detected on a Nunc Maxisorp™ 96-well plate coated with 50 µl of 1 µg/ml uPAR. Fabs (either culture supernatant, periplasmic fraction, or purified protein at 22.5 µg/ml) were applied to the plate’s wells, which were then washed. Bound Fabs were detected using 100 µg/ml of HRP-conjugated anti-myc antibody clone 9E10 (Roche). Three wells not coated with uPAR were included to control for nonspecific Fab binding. For ELISA assays using culture supernatants, bound 9E10-HRP was detected using 1-Step™ Turbo-TMB ELISA (Pierce) for endpoint analysis at 450 nm according to the manufacturer’s protocol. For all other experiments, bound 9E10-HRP was detected as the rate of increase of the absorbance at 650 nm in the presence of the TMB substrate.

**Sequence Analysis.** The heavy and light chain expression cassettes of all 36 uPAR-binding clones were sequenced. The complementarity determining regions (CDRs) of the heavy and light chain sequences were aligned using the ClustalW2 server (25).

**Competitive ELISA.** 95 µl of each Fab was combined with 6 nM high molecular weight uPA (HMW-uPA) (American Diagnostica). The resulting mixture was incubated with the uPAR-coated microplate wells described in the previous section. Wells not coated with uPAR were included to control for any nonspecific binding of HMW-uPA. Wells coated with uPAR and incubated against all Fabs without HMW-uPA were included to control for nonspecific protease activity. Maximal uPA binding was determined by incubating HMW-uPA with uPAR-coated wells, without any Fab. Unbound Fabs and HMW-uPA were removed by washing. The amount of bound HMW-uPA was measured by assaying proteolytic activity in the treated wells using the chromogenic uPA substrate Spectrazyme® UK (American Diagnostica) and monitoring the rate of increase of the absorbance at 405 nm. The wells were further assayed to detect the presence of bound Fab using 9E10-HRP as described in the previous section.

**uPA activity in presence of Fabs.** Fabs were tested for direct inhibition of uPA in two ways. First, 1 µg/ml of HMW-uPA was incubated in uPAR-coated plates; unbound HMW-uPA was removed by washing, and Fabs were added to the wells at 25 µg/ml. The activity of HMW-uPA in the presence and absence of Fab was measured as described above. Second, 10 nM HMW-uPA and low molecular weight uPA (LMW-uPA) (American Diagnostica) were incubated in a microtiter plate in the presence and absence of 450 nM Fab. The activity of HMW- and LMW-uPA was measured in triplicate by assaying proteolytic activity as described above.

**Human IgG1 Antibody Expression and Purification.** Heavy and light chain Fab sequences were amplified by PCR and separately cloned into vector pTT5-SP-H1, a modification of the pTT5 vector (National Research Council of Canada). Heavy and light chain expression vectors were transformed into NEB Turbo Competent *E. coli* (NEB) and large-scale plasmid preparations were performed using the Pure Yield Plasmid Midiprep system (Promega). The sequences of all full-length antibody expression clones were confirmed.

**HEK-293-EBNA1 cells,** a generous gift from Yves Durocher of the Canadian National Research Council, were adapted to GIBCO® FreeStyle™ 293 Expression Medium (Invitrogen) supplemented with 50 µg/ml G418. Heavy and light chain encoding pTT5 plasmids were cotransfected into the cells with jetPEI™ (Polyplus) according to the manufacturer’s protocol. Cells were incubated for four to five days post-transfection, after which the IgG-containing spent media was harvested. IgGs were purified on a Protein A agarose (Pierce) affinity column, eluted with 100 mM citrate pH 3.0, neutralized, dialyzed overnight against PBS, and stored at 4 °C. IgG expression levels were determined using the Easy-
Titer Human IgG Assay Kit (Pierce) and spectrophotometric readings at 280 nm.

Surface Plasmon Resonance. The interaction affinities between uPAR and 1A8, 2B1, 2G10, and 2E9 were determined by equilibrium surface plasmon resonance (SPR) using a Biacore 1000. In order to abrogate the effect of avidity, antibodies were immobilized on the surface of a Biacore CM5 chip and soluble uPAR was flowed as the analyte. Four Biacore CM5 chip flow cells were sequentially treated, according to the manufacturer’s protocol, with 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). 1A8, 2B1, 2E9 and 2G10 IgGs were each diluted to 5 µg/ml in 10 mM sodium acetate pH 5.0 and then immobilized to separate flow cells to obtain approximately 2700 relative response units. The flow cells were blocked with 1 M ethanolamine pH 8.5 after antibody immobilization. A flow cell on each CM5 chip was immediately treated with 1 M ethanolamine pH 8.5 after antibody immobilization. A flow cell on each CM5 chip was immediately treated with 1 M ethanolamine pH 8.5 after antibody immobilization. A flow cell on each CM5 chip was immediately treated with 1 M ethanolamine pH 8.5 after antibody immobilization. A flow cell on each CM5 chip was immediately treated with 1 M ethanolamine pH 8.5 after antibody immobilization.

Soluble human uPAR was injected over flow cells at the following concentrations: 450 nM, 225 nM, 112.5 nM, 56.25 nM, 28.13 nM, 14.1 nM, 7 nM, 3.5 nM, 1.8 nM, and 0 nM. Bound uPAR was removed with 10 mM glycine pH 1.5. Instrument response values were recorded and imported into Scrubber2 (BioLogic Software) for analysis. Data were normalized using the double referencing method (26), and analyzed using a one site binding model as implemented in Scrubber2. Response values reached a stable plateau as judged by a change of less than 0.05% over the last minute of injection.

Flow Cytometry. A confluent flask of either HEK 293 cells or HEK 293 uPAR cells was treated with TrypLE Express (Gibco). Harvested cells were re-suspended in Stain Buffer (BD Pharmingen) and either 5x10⁵ or 1x10⁶ cells were transferred to tubes for antibody staining. 1A8, 2B1, 2E9, 2G10, and whole human IgG (Sigma) were added to a final concentration of 5 µg/ml. 2G10 and 3C6 Fab were added to a final concentration of 50 µg/ml. All samples were incubated on a rotator at 4 °C for 30 minutes after addition of antibody, harvested by centrifugation, and resuspended in 500 µl of Stain Buffer. The IgG samples were resuspended and incubated with 20 µl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-human monoclonal antibody (BD Pharmingen), while the Fab samples were incubated with Alexa Fluor® 488-conjugated mouse anti-cMyc monoclonal antibody (AbD Serotec). 5x10⁵ cells were analyzed with a Beckton Dickinson FACSCalibur cytometer. Data analysis was performed with FlowJo version 7.2.4.

Adhesion Assay. The cell adhesion assay was performed as described previously (14). Briefly, H1299 cells (2x10⁴) were seeded onto fibronectin (FN)-coated (10 µg/ml) or vitronectin (VN)-coated (5 µg/ml) plates with or without the anti-uPAR Fabs (10 µg/ml), RGD peptide, or RAD peptide (0.4 mM). Attached cells were fixed with methanol and Giemsa stain was used for colorimetric analysis by measuring the optical density at 550 nm. FN and VN were purchased from Sigma-Aldrich (St. Louis, MO). RGD and RAD peptides were purchased from Anaspec (San Jose, CA).

ERK Phosphorylation Assays. Serum-starved H1299 cells were washed with 50 mM glycine-HCl, 100 mM NaCl, pH 3.0 for three minutes to remove surface-bound endogenous uPA, and neutralized with 0.5 M HEPES, 0.1 M NaCl pH 7.5 for 10 minutes on ice. Cells were pre-treated with 10 µg/ml of 1A8, 2B1, 2E9, 2G10, or control human IgG (each 10 µg/ml) for one hour at 37 °C. Pro-uPA was added to 10 nM and incubated at 37 °C for five minutes to initiate ERK activation. After incubation, cells were lysed in RIPA buffer (Pierce) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich) and blotted for phospho- and total ERK (Cell Signaling). In the case of FN-stimulated ERK phosphorylation, cells were cultured on a FN- (10 µg/ml) coated surface for 30 minutes before lysis.

Invasion Assays. H1299 human lung cancer cells (1x10⁵) were pre-treated with 1A8, 2B1, 2E9, 2G10, or control human IgG (each 10 µg/ml), and 2G10, 3C6, 2G10+3C6 Fab (5-10 µg/ml) for one hour at 37 °C. Cells were then seeded into BD Biocoat™ Invasion Chambers (BD Biosciences) with Matrigel, Collagen I, or Matrigel/Collagen I mix-coated tops and FN pre-coated bottoms, and then cultured overnight in serum-free DMEM containing 5 mg/ml BSA; fetal bovine serum was added to the lower chamber at 5%. 24 hours later, the matrices and cells on the membrane’s top chamber side were removed, and cells on the membrane’s bottom chamber side
were fixed with methanol, stained with Giemsa, extracted in 10% acetic acid, and measured in a plate reader at 595 nm. All assays were performed in triplicate and the data expressed as percent inhibition by the antibodies:

\[
\text{%Inhibition} = \left[ \frac{(OD_{\text{Ctrl}} - OD_{\text{Test}})}{OD_{\text{Ctrl}}} \right] \times 100
\]

Acid-extracted rat-tail collagen I was purchased from Sigma (Catalog # C7661) and was reconstituted according to the manufacturer’s protocol.

**Anti-uPAR Co-immunoprecipitation.**

H1299 cells (1x10^7) were lysed in Triton lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl and 1% Triton X-100) supplemented with protease inhibitors (Sigma) and 1 mM PMSF. Clarified lysates were first incubated with anti-uPAR Fabs (10 µg/ml) at 4 °C for 1 hour, then with Penta-His Antibody (Qiagen) for 1 hour, and finally with 50 µl of mixed Protein A and Protein G Agarose beads overnight. The immunoprecipitates were subjected to SDS-PAGE and Western blot analysis for uPAR and α5 integrin. The anti-uPAR monoclonal antibody (R2) was a kind gift from Michael Ploug (Finsen Lab, Copenhagen, Denmark). The anti-α5 integrin polyclonal antibody was purchased from Chemicon (Temecula, CA).

**uPAR and MMP Invasion Assay-** H1299 human lung cancer cells (1x10^7) were pre-treated with 2G10 or 3C6 Fab (5 µg/ml), and either GM6001 (0.1 µM, Chemicon), or anti-MT1-MMP catalytic domain (5 µg/ml, Clone 3G4.2, Millipore) for one hour at 37 °C. Cells were then seeded into BD Biocoat™ Invasion Chambers (BD Biosciences) with Matrigel/Collagen I mix-coated tops and FN pre-coated bottoms, and then cultured overnight in serum-free DMEM containing 5 mg/ml BSA; media containing fetal bovine serum was added to the lower chamber at 5%. Length of invasion, fixation, staining, and analysis was done exactly as described for the previously documented invasion assays.

**RESULTS**

**Phage display identifies uPAR binding Fabs.** Prior to panning, the binding of active uPAR to a microplate surface was confirmed by detecting the binding of HMW-uPA to the uPAR-coated surface. Binding of HMW-uPA was detected by assaying for the presence of specific proteolytic activity within a microplate well with the uPA substrate spectrazyme UK (data not shown) after incubating the uPAR-coated plate with HMW-uPA and stringently washing.

Fabs capable of binding human uPAR were obtained after three rounds of panning, in which washes to remove weakly bound Fab-displaying phage were made increasingly stringent. 384 independent clones were evaluated from the final round of panning. To confirm that these Fabs could be expressed in bacteria, culture supernatants (into which a small fraction of Fabs accumulated after IPTG addition) were tested for the presence of Fab capable of binding to uPAR. From these 384 clones, 96 were selected for further analysis on the basis of reproducible uPAR binding. Periplasmic protein fractions were then prepared from the 96 clones. With these fractions, ELISA analyses gave stronger, more consistent signals compared to that of culture supernatants, presumably due to the higher concentration of Fab in the periplasm as compared to culture supernatant. Of the 96 clones, 36 candidates were confirmed as strong binders of uPAR, with an average signal greater than 8-fold over background (data not shown).

**Sequence analysis and small-scale expression identifies unique Fabs.** The 36 candidates were sequenced and evaluated for expression at the 100 ml culture scale. Sequencing of the heavy and light chain expression cassettes revealed that 22 of the 36 candidates have unique Fab sequences. ClustalW alignment of these sequences yielded a percent identity dendrogram with two distinct groups of antibodies defined by having a κ or λ light chain (Figure 1A). Several sub-groups of highly related sequences are evident within the κ light chain group whereas eight antibodies with a relatively low degree of sequence similarity are evident within the λ light chain group. Alignment of the six complementary determining regions (CDRs) of each unique Fab (Figure 1B) shows that the CDR sequences determine the subgrouping pattern observed in the dendrogram of Figure 1A. The lowest pair wise sequence identity between antibody CDRs is 22%.

The expression levels of the 22 unique Fabs in *E. coli* were determined after IPTG induction of 100 ml cultures. Histidine-tagged
Fabs from the periplasmic fraction were obtained by osmotic shock, purified on a nickel chelating Sepharose column, and analyzed for expression by Western blot. Two Fab clones showed no expression and were not pursued further (Figure 1B). Small-scale expression of the remaining Fabs, with the exception of 2E9, yielded 250 µg/L of E. coli culture. Fab 2E9 expression yields were five fold lower.

Purified Fabs were further characterized by uPAR ELISA. Initial measurements of bound antibody exhibited a large variance between different Fabs, but control experiments measuring uPA binding to immobilized uPAR did not show similar variance suggesting that these differences reflect inherent disparities in binding mode or affinity between different Fabs (data not shown).

Clustering individual clones based on their sequences and bacterial expression abilities narrowed the list of Fabs to further pursue. Sequences with a sequence similarity greater than or equal to 82% were clustered together (Figure 1). From these groupings, a representative clone demonstrating robust small-scale expression was chosen, thus narrowing the list of Fabs to 12 clones for further analysis.

**Competitive ELISA identifies 2E9 and 2G10 as the most competitive with uPA for uPAR binding.** Purified Fabs from the 12 remaining clones were analyzed for their ability to compete with uPA for binding to immobilized uPAR. The presence of uPA was measured by the amount of bound proteolytic activity in the presence and absence of each Fab (Figure 2). This assay identified 2E9 and 2G10 as competitors of the uPA/uPAR interaction. Controls showed that these antibodies did not directly inhibit uPA’s proteolytic activity (data not shown).

The competitive ELISA data also suggested that 1A8 and 2B1 do not compete with uPA for uPAR binding. To verify that these Fabs were not weak uPA competitors, the ratio of bound Fab in the presence of uPA to bound Fab in the absence of uPA was calculated (Figure 2, inset). The amount of Fab bound in the presence and absence of uPA was determined in the same uPAR coated well, therefore some loss of Fab is expected due to processing between measurements. This assay verified that 1A8 and 2B1 bound a non-uPA binding site on uPAR. The two strongest non-competitive binders, 1A8 and 2B1, and the two strongest competitive inhibitors, 2G10 and 2E9, were chosen for further analysis.

**Full-length IgG expression in mammalian cells produces reagent quantities of antibody.** The heavy and light chain sequences of 1A8, 2B1, 2G10, and 2E9 were cloned into the mammalian expression vector pTTS-SPH1 for high-level expression by transient transfection in HEK-293-EBNA1 cells (Figure 3). Co-transfection of varying ratios of heavy and light chain expression plasmids revealed that an equal mass of heavy and light chain DNA, which corresponds to a slight molar excess of light chain plasmid particles, produced the highest level of antibody. A total DNA: PEI ratio of 1 µg: 4 µl, and sub-confluent maintenance of HEK 293-EBNA1 cells resulted in greater than 90% transfection efficiency. Optimal time to harvest post transfection was four to five days. Antibody expression yield was sequence dependant and varied between 20 mg/L to 100 mg/L of culture supernatant at the 1 ml scale, and between 10 mg/L and 50 mg/L in large scale trials (500 ml).

**Surface plasmon resonance reveals low nM affinities for uPAR.** The monovalent interaction affinity between uPAR and the antibodies 1A8, 2B1, 2G10, and 2E9 were determined by equilibrium surface plasmon resonance methods using a Biacore 1000. Analysis of instrument response versus analyte (uPAR) concentration yielded monovalent dissociation constants in the nanomolar range (Figure 4).

**Flow cytometry shows specific labeling of uPAR expressing cells.** The ability of the identified antibodies to bind uPAR, as it is presented on the cell surface, was analyzed by flow cytometry. HEK-293 cells stably expressing membrane-bound human uPAR were labeled with full-length anti-uPAR IgGs, or an isotype control. Anti-uPAR IgGs were detected with an anti-human Fc FITC-conjugated secondary antibody. Labeled cells were analyzed on a flow cytometer (Figure 5). All the antibodies tested indicated robust labeling of uPAR-expressing HEK-293 cells, but did not show labeling of the parental HEK-293 cells lacking uPAR expression indicating highly specific binding (data not shown).

2E9 and 2G10 decrease H1299 invasion. H1299 cells have also been shown ex vivo to migrate through, or invade, extracellular matrix components such as Matrigel in a manner that is
dependent on uPA binding to uPAR (19). A strong ex vivo Matrigel invasion phenotype is thought to correlate with the metastatic potential of a cancer cell in vivo. Analysis of the effects of antibodies 1A8, 2B1, 2G10, and 2E9 on Matrigel invasion by H1299 cells, which produce uPA in an autocrine fashion, shows that 2G10 and 2E9 are both capable of inhibiting migration, whereas 1A8 and 2B1 are not (Figure 6A).

2E9 and 2G10 decrease uPA-dependent ERK phosphorylation in H1299 cells. The human lung cancer cell line H1299 exhibits pro-proliferative ERK phosphorylation and activation that is dependent on signaling events mediated by binding of uPA to uPAR (19). This cell line was used to test the ability of the anti-uPAR antibodies to inhibit uPAR-dependent pro-proliferative signals triggered by uPA binding. The results demonstrate that antibodies 1A8 and 2B1 do not inhibit ERK phosphorylation under the conditions tested. However, 2E9 and 2G10, which compete with uPA binding to uPAR, are able to inhibit ERK phosphorylation (Figure 6B).

3C6 decreases FN-dependent ERK phosphorylation in H1299 cells, and abrogates their FN- and VN-dependent adhesion. The activation of FN-dependent ERK phosphorylation in H1299 cells is dependent on the formation of the uPAR/α5β1/FN complex (19). To determine if any of the unique anti-uPAR Fabs interfere with the uPAR/α5β1 interaction, we tested their ability to decrease ERK phosphorylation in H1299 cells seeded in FN-coated wells. We identified 3C6 as able to significantly decrease FN-dependent ERK phosphorylation (Figure 7A).

To further characterize the functional effects of 3C6, a FN adhesion assay was utilized. The α5β1/FN interaction can occur in a uPAR-independent context that is sensitive to antagonism by the RGD peptide, and in a uPAR-dependent context that is resistant to the RGD peptide (14). In the presence of both the RGD peptide and 3C6, H1299 adhesion to FN-coated wells was abrogated (Figure 7B). The selectivity of this effect was verified by inclusion of RAD peptide and the Fab form of the uPA competitor, 2G10, as negative controls.

To determine if 3C6’s ability to disrupt uPAR/β1-mediated adhesion is general, we characterized the ability of uPAR/α3β1-mediated H1299 cell adhesion to VN. In an assay similar to the FN adhesion assay, it was found that 3C6 could also prevent the adhesion of H1299 cells to VN in the presence of RGD peptide (Figure 7B), suggesting that 3C6 is able to specifically block the functions of uPAR complexes with multiple β1 integrins.

3C6 Fab binds to uPAR over-expressing HEK cells. To confirm that 3C6 recognizes uPAR as displayed on a cell’s surface, we utilized the same flow cytometry assay used to characterize the anti-uPAR IgGs. Since the investigation of 3C6-dependent cellular effects was done with the Fab form of the antibody, we continued to use this format for flow cytometry. We also included the 2G10 Fab as a benchmark for an antagonistic antibody’s ability to bind cellular uPAR-expressing HEK-293 cells. The data indicate that the 3C6 Fab can bind to cells that over-express uPAR, albeit not as robustly as the 2G10 Fab (Figure 8A).

3C6 prevents the association of uPAR and β1 integrins in H1299 cells. α3β1 and α5β1 are the major β1 integrins that associate with uPAR in H1299 cells (14). To determine if 3C6 directly blocks uPAR’s association with β1 integrins, 3C6 and 2G10 Fabs were used to immunoprecipitate uPAR, and uPAR ligands, from H1299 lysates. The resulting immunoprecipitates were analyzed by Western blot for uPAR, α5, and β1 integrin subunits. 2G10 appears to be able to bind uPAR at the same time as β1 integrin (Figure 8B). The absence of β1 in the 3C6 immunoprecipitation suggests that 3C6 prevents uPAR/β1 association. Since α5β1 integrin association with uPAR is known to effect changes in ERK phosphorylation and MMP expression (14), we also probed for the presence of the α5 subunit. The results indicate that 2G10 is able to bind uPAR simultaneously with α5β1 integrin, while 3C6 prevents uPAR’s association with α5β1 integrin (Figure 8C).

2G10 and 3C6 show a combined effect on inhibition of H1299 invasion through cross-linked matrices. Migration is a complex phenomenon that requires modulation of adhesion and degradation of ECM (27). As shown in Figure 6A, antagonism of the uPAR/uPA complex by 2E9 and 2G10 inhibits the invasion of H1299 cells. To determine if 3C6 has a similar effect on invasion by antagonizing the uPAR/β1 complexes, we
explored whether the 3C6 Fab could have an additional effect on invasion inhibition when used simultaneously with the uPA competitor Fab, 2G10, to block cell invasion through Matrigel/Collagen I or Collagen I. As shown in Figure 9A, not only do 2G10 and 3C6 Fabs inhibit invasion through Collagen I, but a combined dosage exhibits a combined response.

Additionally, the invasion assay was repeated on a substrate comprised of both Matrigel and Collagen I, to provide a matrix that contains more physiologically relevant physical cues for migration and ECM degradation. The results are consistent with the increased response observed on the collagen I coated inserts (Figure 9B).

2G10 and 3C6 do not confer additional invasion inhibition above MMP inhibitors. Figure S1 shows the invasion inhibition observed for the different treatments of H1299 cells. The concentrations were chosen below the saturation point of the anti-uPAR antibodies (as can be gauged by the invasion data from Figure 9), so as to provide a preliminary indication as to whether additional invasion inhibition was conferred by using two different inhibitors. Additional treatment of MMP-inhibited H1299 cells (which were treated with either broad spectrum MMP inhibitor GM6001, or the antibody that was specific for the MT1-MMP catalytic domain) with anti-uPAR antagonist antibodies 2G10 or 3C6 did not confer invasion inhibition that was significantly greater than treatment with MMP inhibitors alone.

DISCUSSION

uPAR-mediated signaling has been implicated in tumor cell invasion, survival, and metastasis (28). Many of these signaling events are dependent on binding extracellular ligands such as uPA and integrins. Therefore, the identification of reagents with favorable pharmacokinetic characteristics capable of binding to and interfering with uPAR-mediated signaling is an area of great interest.

Here we report Fabs from a highly diverse and naïve human Fab phage display library that are capable of binding uPAR. Antibodies that inhibit the binding of the known uPAR ligands uPA and β1 integrins were produced. These antibodies are able to robustly label membrane-bound uPAR expressing cells and inhibit uPAR-mediated signal transduction and cellular migration.

The identified anti-uPAR antibodies can be broadly categorized into two different groups by heavy chain sequence similarity. Fourteen of the antibodies show significant heavy chain CDR sequence similarity (2G10 through 2B7 in Figure 1B), while the remaining eight do not. 2G10, 2B1, and 1A8 have similar heavy chain CDRs, but 2G10 competes with uPA for uPAR binding, while 2B1 and 1A8 do not. Interestingly, the light chain CDRs of 1A8 and 2B1 are highly similar to one another, and different from that of 2G10, indicating that the light chain may play a role in 2G10’s ability to compete with uPA for uPAR binding. 2E9, which also competes with uPA, has heavy and light chain CDRs that are highly dissimilar to 1A8, 2B1, or 2G10. This suggests that there is more than one method of interfering with uPAR/uPA binding. To corroborate this supposition, the flow cytometry data (Figure 5) imply that even though 2E9 and 2G10 both compete with uPA for uPAR binding, they recognize cellular uPAR differently.

3C6 has unique light and heavy chain CDRs when compared with 1A8, 2B1, 2G10, and 2E9, consistent with its role as the sole anti-uPAR antibody that antagonizes the uPAR/β1 interactions. The diversity of the anti-uPAR antibody sequences suggests that they may possess multiple uPAR binding modes and may antagonize other uPAR/ligand interactions.

There are several instances where the binding affinity of the antibody for uPAR does not predict functional efficacy in cell-based assays. The monovalent binding affinity of the 2G10 and 2E9 IgG antibodies are weaker than the affinity between uPAR and uPA (0.31 nM) (29), yet these IgGs inhibit uPAR-mediated invasion. One explanation for this phenomenon is that the avidity conferred by the bivalent IgG forms of 2E9 and 2G10 increases their apparent affinity for uPAR, thus allowing competition; however, the functional effect of the 2G10 IgG and Fab are comparable (Figures 6A and 9), suggesting that IgG avidity alone cannot explain the functional efficacy of these antibodies. Additionally, while 3C6 appears to bind uPAR over-expressing cells less efficiently than 2G10 (Figure 8A), it is still able to affect pERK levels, adhesion, and invasive potential in
H1299 cells. The same can be said for 2E9, which exhibits uPA competition. While SPR measurements show 2E9 to have the highest monovalent affinity for uPAR, flow cytometry data indicate that it binds to uPAR over-expressing HEK-293 cells less efficiently than 2G10; however, 2E9 inhibited H1299 invasion more so than 2G10 (Figure 6A). Thus, in this investigation, SPR and flow cytometry provide a means to determine uPAR recognition in vitro and ex vivo, but do not predict functional efficacy in cell-based assays.

Characterization of these antibodies has shown their antagonism to be highly selective. 2G10 and 2E9 inhibit uPA binding to uPAR while 3C6 inhibits β1 integrin binding to uPAR. These antibodies are also capable of inhibiting processes mediated by uPA/uPAR or uPAR/β1 complexes in the lung cancer cell line H1299, such as Matrigel/Collagen I or Collagen I invasion, and ERK phosphorylation. In contrast 1A8 and 2B1 do not show any inhibition of uPA binding, invasion, or ERK phosphorylation, and 2G10 does not abrogate neither FN- nor VN-mediated adhesion of H1299 cells (Figure 7B-C). These results suggest the high degree of functional selectivity of these antibodies. Flow cytometry experiments show that these antibodies specifically bind to cells that express uPAR on their extracellular surface without detectably labeling control cells. Furthermore, they exhibit no binding to mouse uPAR (data not shown). The lack of reactivity towards mouse uPAR suggests that these antibodies would be ideal for studying inhibition of tumor growth and metastasis in mouse xenograft models that use human cell lines. In addition, since these antibodies are fully human, antibody-dependent cell-mediated cytotoxicity would not contribute to any anti-proliferative or anti-metastatic effects observed in mouse models.

A promising observation is that combined 2G10 and 3C6 treatment of H1299 cells has a combined effect on inhibiting invasion through Matrigel/Collagen I and Collagen I matrices (Figure 9). Doubling the concentration of 2G10 or 3C6 does not lead to a two-fold increase in invasion inhibition. This could be ascribed to saturation of uPAR-mediated invasion pathways; however, a slightly greater invasion inhibition is achieved than from doubling either antibody concentration (Figure 9). While further experiments are required to pinpoint the specific molecular mechanisms contributing to this phenomenon, as well as the type of relationship that combined treatment with 2G10 and 3C6 exhibits, it is evident that combined treatment with a uPAR/uPA antagonist antibody and a uPAR/β1 antagonist antibody exhibits a different cellular effect than treatment with only one antibody antagonist.

Previous investigations into the ability for cancer cells to migrate through matrices that more closely recapitulate the basement membrane matrix have shown that membrane type-1, type-2, and type-3 metalloproteinases (MT1-, MT2-, and MT3-MMPs) to be essential for invasion (30). In particular, MT1-MMP has been implicated as a downstream factor that determines proteolytic invasion through basement membranes directly and indirectly (31). Previous work indicated that disruption of the uPAR/β1 interaction leads to down regulation of MMP1 and MMP9 transcription, which leads to lower protease activity (19). Therefore, we were inclined to accept that uPAR’s interaction with uPA and β1 integrins ultimately lead to the activation of matrix-degrading MMPs. Additionally, the uPAR/uPA interaction has been suggested to be involved in the activation of ECM-degrading MMP2 (32). In order to resolve whether uPAR contributed to invasion beyond that of MMPs, MT1-MMP in particular, we conducted another invasion assay where we co-treated H1299 cells with either 2G10 or 3C6, and the broad-spectrum MMP inhibitor GM6001 or an antibody against the catalytic domain of MT1-MMP. We observed that neither 2G10 nor 3C6 contributed any additional invasion inhibition above that seen in GM6001 or anti-MT1-MMP treated samples. This suggested that uPAR-mediated invasion is ultimately determined by MMP activity, particularly that of MT1-MMP (Supplementary Figure S1).

The expression of MT1-MMP, uPAR, and uPA has been previously demonstrated, and implicated in the ECM remodeling of cells (33,34). Additionally, uPAR and MT1-MMP expression has been correlated with an invasive phenotype, particularly in pancreatic cancer cells (35,36). As indicated in Figure S1, MMPs may play the ultimate role in dictating cancer cell invasiveness through cross-linked matrices. While
this would suggest that targeting membrane-type MMPs for therapeutic purposes would be beneficial, MMP inhibitors have met with limited success as cancer therapeutics, likely due to inhibition of MMPs not involved in disease processes (37). Our data suggest an alternate modality for the inhibition of MMP activity, which bypasses much of the problems encountered with the broad-spectrum MMP inhibitors.

The implication for therapeutic development is two-fold: 1) intracellular delivery of uPAR-targeted therapeutics is not required to have a significant effect, and 2) the targeting of two distinct sites on uPAR makes it less likely for resistance mutations to emerge in a population of cancer cells.

In summary, the functional selectivity and specificity of these antibodies suggests that they may be useful for imaging and/or therapeutic purposes in human tumors associated with high levels of uPAR expression. In addition, these antibodies will allow for further mechanistic dissection of uPAR signaling by enabling experiments that concurrently and selectively antagonize multiple uPAR/ligand interactions.

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REFERENCES


**FOOTNOTES**

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The abbreviations used are: BSA, Bovine serum albumin; B-HBST, 0.1% BSA in HBS and 0.05% Tween 20; B-PBST, 5% BSA in PBST; CDR, Complementarity determining region; DI, Domain I; DII, Domain II; DIII, Domain III; ECM, Extracellular matrix; EDC, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; Fab, Fragment antigen binding; FITC, Fluorescein isothiocyanate; HBS, HEPES buffered saline pH 7.0; HMW-uPA, High molecular weight uPA; HRP, Horse radish peroxidase; IgG, Immunoglobulin G; IPTG, Isopropyl β-D-1-thiogalactopyranoside; LMW-uPA, Low molecular weight uPA; M-PBST, 5% non fat dried milk in PBST; NHS, N-hydroxysuccinimide; PBS, Phosphate buffered saline pH 7.4; PBST, PBS and 0.05% Tween-20; Sf9, *Spodoptera frugiperda* 9; SPR, Surface plasmon resonance; Tris, Tris(hydroxymethyl)aminomethane; uPA, Urokinase plasminogen activator; uPAR, Urokinase plasminogen activator receptor; RGD, arginine-glycine-aspartic acid; FN, fibronectin.

**FIGURE LEGENDS**

**Figure 1:** Sequence homology for uPAR-binding Fabs identified by phage display. A. The heavy and light chain protein sequences of the 22 unique clones were aligned to generate a percent identity tree diagram. The number of identical clones is indicated in parentheses for redundant Fab sequences. The Fab subgroups, defined by their light chain identity (κ or λ), are labeled. The vertical line indicates the 82% sequence identity threshold. Sequences that branch to the right of the 82% cut-off are considered equivalent. B. The sequences of the CDR loops of each unique Fab were aligned and shaded to indicate sequence identity. The name of each CDR loop is indicated above the alignment. Fab heavy and light chain protein sequences with greater than 82% sequence identity were grouped together (box). A representative clone was selected from each group based on expression levels in *E. coli* Rosetta-gami™ B cells and is indicated to the left of the box. Asterisks indicate Fab clones that did not express in Rosetta cells.

**Figure 2:** Binding of uPA to uPAR in the presence of Fab. uPA was added to a uPAR-coated plate in the absence and presence of each Fab. The presence of uPA was determined by the amount of bound proteolytic activity, and is reported as the initial velocities from the progress curves. Maximal uPA binding was determined by incubating uPA without Fab and is labeled “no Fab”. Data is plotted left to right from Fabs that do not compete with uPA for uPAR binding to Fabs that show maximal competition. Inset. For 1A8 and 2B1, the amount of Fab bound to uPAR in the presence and absence of uPA was
determined by ELISA. The ratio of bound Fab in the presence of uPA to bound Fab in the absence of uPA is reported as a percentage.

**Figure 3: IgG expression by transient transfection.** A. Fab sequences were grafted onto an IgG1 scaffold by independently sub-cloning the heavy and light chain sequences into pTT5-SP-H1. The plasmid map of this transient expression vector is shown. For a given antibody, both the pTT5-SP-H1-heavy chain vector and pTT5-SP-H1-light chain vector were co-transfected into HEK-293-EBNA1 cells for expression. B. SDS-PAGE analysis of purified antibodies is shown. The \( \lambda \) light chain of 2E9 runs at a higher apparent molecular weight than the \( \kappa \) light chain of the other antibodies.

**Figure 4: Equilibrium affinity determination of uPAR antibody interaction.** Percent of maximal surface plasmon resonance response during analyte (uPAR) injection versus analyte concentration is shown. Curve fitting for 2E9 (open circle), 1A8 (open square), 2G10 (closed diamond), and 2B1 (x) yielded \( K_D \) values that are summarized in the table.

**Figure 5: Detection of cell surface uPAR with human anti-uPAR antibodies.** (A-D), white profiles represent staining with control whole human IgG; shaded profiles represent staining with human anti-uPAR antibody. The identity of the human anti-uPAR antibody is indicated within the shaded profile (A = 1A8, B = 2B1, C = 2E9, D = 2G10). To quantify the relative staining intensities of the human anti-uPAR antibodies, the same gate (horizontal line) was applied to each sample. The % of cells staining positive for uPAR expression is indicated above the gate.

**Figure 6: Inhibition of uPA/uPAR mediated invasion and signaling in H1299 cells.** (A) H1299 cells were pre-treated with antibodies (10 \( \mu \)g/ml): 2E9, 2G10, 2B1, and 1A8 before they were allowed to invade Matrigel for 24 hours. The cells that migrated through and attached to the bottom of the filter were fixed, stained with Giemsa, and extracted with 10% acetic acid. Cell invasiveness is evaluated by measuring \( OD_{595} \) nm. The results are expressed as percent inhibition of that observed with no treatment control. (B) H1299 cells expressing endogenous uPAR were serum-starved, acid washed, pre-treated with antibodies (10 \( \mu \)g/ml), and then incubated with pro-uPA (10 nM). The lysates were immuno-blotted with anti-pERK (top panel) and anti-total ERK (bottom panel).

**Figure 7: Determination of 3C6 as a putative uPAR/\( \beta_1 \) integrin antagonist.** (A) H1299 cells were serum-starved, acid washed, were pre-treated with Fabs (10 \( \mu \)g/ml): 2B1, 2B7, 2B11, 2D5, 2E9, 2G10, 2G12, 3C6, and 4C1, and cultured on a FN-coated surface (10 \( \mu \)g/ml) for 30 minutes before lysis. The lysates were immuno-blotted with anti-pERK (top) and anti-total ERK (bottom). (B) H1299 cells were seeded on FN-coated (10 \( \mu \)g/ml) or VN-coated (5 \( \mu \)g/ml) 96-well plates with or without anti-uPAR antibody, and RGD or RAD peptide. Shown here is a direct comparison between 2G10 (uPAR/uPA antagonist) and 3C6, now identifiable as an uPAR/\( \beta_1 \) integrin antagonist. (C) A normalized graph comparing the adhesion for each antibody treatment on the two different ECM coatings, obtained by dividing the average reading from RGD-treated wells by that from RAD-treated wells. Note that 3C6 treatment disrupts uPAR-mediated integrin adhesion at least four fold more than 2G10 treatment.

**Figure 8: 3C6 binds to cell surface uPAR and abrogates uPAR association with \( \alpha_5\beta_1 \) integrin.** (A) HEK 293 cells over-expressing uPAR were stained with 3C6 and 2G10 to confirm 3C6’s ability to bind cell surface uPAR. The dashed white profile represents staining with 2G10 Fab; the shaded profile represents staining with 3C6 Fab; the solid white profile represents no Fab staining, but inclusion of the AlexaFluor 488 conjugated secondary. (B-C) H1299 lysates were incubated with anti-uPAR Fab (2G10 or 3C6), Penta-His antibody, and Protein A/G agarose. The presence of \( \beta_1 \) integrin (B) or \( \alpha_5 \) integrin (C) and uPAR were probed with the respective antibodies in Western analysis.
Figure 9: Combined 2G10 and 3C6 treatment of H1299 cells significantly decreases invasive potential through Matrigel/Collagen I and Collagen I. H1299 cells were pre-treated with antibodies (2G10, 3C6, and 2G10/3C6 at 5-10 µg/ml) before seeding on the Collagen I-coated (A) or Matrigel/Collagen I-coated (B) top membrane of a 24-well Transwell plate (10^5 cells/well in triplicate). Cells were incubated for 24 hours. The cells that migrated through and attached to the bottom of the filter were fixed, stained with Giemsa, and extracted with 10% acetic acid. Cell invasiveness is evaluated by measuring OD_{595 nm}. The results are expressed as a percentage of inhibition observed in the no treatment control. Note, the inhibition potential of combined 2G10 (5 µg/ml) and 3C6 (5 µg/ml) is significantly stronger than either antibody alone (10 µg/ml), suggesting an additive effect.
Figure 2
Figure 3

A. [Diagram of pTT5-SP-H1 vector with restriction sites and functional elements labeled, such as SalI, NheI, NotI, IgG HC Const, Poly A, CMV, OriP, pMB1ori, and AmpR.]

B. [Image of gel electrophoresis with DNA bands labeled as ladder, 1A8, 2B1, 2E9, and 2G10, and molecular weight markers in kDa ranging from 70 to 25.]
Figure 4

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<th>2E9</th>
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<td>53.5+/-0.2</td>
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</tbody>
</table>
Figure 5
Figure 6

A. % Inhibition

B. Treatment: IgG, 2E9, 2G10, 2B1, 1A8

H1299 Cells

P-ERK

T-ERK
Figure 7

A.

B.

C.
Figure 9

A. Invasion on Collagen I

P = 0.03

\[
\begin{array}{cccccc}
\text{Antibody} & \text{2G10} & \text{2G10} & \text{3C6} & \text{3C6} & \text{2G10/3C6} \\
\mu g/ml & 5 & 10 & 5 & 10 & 5/5 \\
\end{array}
\]

B. Invasion on Matrigel/Collagen I

P = 0.003

\[
\begin{array}{cccccc}
\text{Antibody} & \text{2G10} & \text{2G10} & \text{3C6} & \text{3C6} & \text{2G10/3C6} \\
\mu g/ml & 5 & 10 & 5 & 10 & 5/5 \\
\end{array}
\]