Inhibitory monoclonal antibodies against mouse proteases raised in gene-deficient mice block proteolytic functions in vivo

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Identification of targets for cancer therapy requires the understanding of the in vivo roles of proteins, which can be derived from studies using gene-targeted mice. An alternative strategy is the administration of inhibitory monoclonal antibodies (mAbs), causing acute disruption of the target protein function(s). This approach has the advantage of being a model for therapeutic targeting. mAbs for use in mouse models can be obtained through immunization of gene-deficient mice with the autologous protein. Such mAbs react with both species-specific epitopes and epitopes conserved between species. mAbs against proteins involved in extracellular proteolysis, including plasminogen activators urokinase plasminogen activator (uPA), tissue-type plasminogen activator (tPA), their inhibitor PAI-1, the uPA receptor (uPAR), two matrix metalloproteinases (MMP9 and MMP14), as well as the collagen internalization receptor uPARAP, have been developed. The inhibitory mAbs against uPA and uPAR block plasminogen activation and thereby hepatic fibrinolysis in vivo. Wound healing, another plasmin-dependent process, is delayed by an inhibitory mAb against uPA in the adult mouse. Thromboembolism can be inhibited by anti-PAI-1 mAbs in vivo. In conclusion, function-blocking mAbs are well-suited for targeted therapy in mouse models of different diseases, including cancer.

Keywords: mouse monoclonal antibodies, extracellular proteolysis, collagen internalization, in vivo models, plasminogen activation

INTRODUCTION

Most of our understanding of the in vivo roles of extracellular proteases, their cellular receptors and inhibitors is derived from studies using gene-targeted mice. The phenotype of a gene-deficient mouse can, however, be influenced by redundancy, when the function of the protein encoded for by the inactivated gene is compensated due to overlapping functions between proteins (Page-McCaw et al., 2007; Stevens et al., 2012). Identification of the in vivo function of a protease/receptor in an adult mouse can be obtained by administration of inhibitory monoclonal antibodies (mAbs), causing acute disruption of the target protein function(s), further offering the advantage of directly serving as a model for therapeutic targeting. In order to avoid immunogenicity effects in mice, the mAbs used should be of murine origin. Development of such mAbs requires immunization of gene-deficient mice with the autologous target protein.

Generation of mAbs against murine proteases using gene-deficient mice was first described by Declerck et al. (1995a). Mice lacking the gene encoding either of the two main plasminogen activators, i.e., tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), were immunized with murine tPA (mtPA) and muPA, respectively. Sera from the immunized mice had high titers of anti-mtPA and anti-muPA antibodies, while similar immunizations of wild-type mice resulted in no or little reactivity toward mtPA and muPA, consistent with these proteins being self-antigens in wild-type mice (Opdenakker et al., 2003).

In this review, the properties of mouse mAbs, all generated by use of gene-deficient mice, will be described with respect to specificity, epitope location, cross-reactivity with the homologous protein from other species (conserved epitopes), and inhibitory function in vitro and in vivo. The antibody-mediated in vivo effect will be compared with that observed in gene-deficient mice to reveal similarities or changes between acute disruption of function in the adult animal and genetic disruption in utero.

MONOClonAL ANTIBOdIES AGAiNS MURINE PLASMINOGEN ACTIVATORS

To generate mAbs against mtPA, mice were immunized twice and subsequently boosted with 10 μg recombinant mtPA before spleen cell isolation and fusion to myeloma cells. Hybridomas were selected for their ability to secrete antibodies reacting with mtPA using ELISA, resulting in the isolation of 203 hybridomas from two fusions. The amount of coating antigen in the ELISA was rather high (200 ng mtPA/well), allowing antibodies with relatively low
affinity to be isolated. Of the 203 mAbs, 21 were selective for mouse tPA, while the residual mAbs cross-reacted with tPA from one or more species, i.e., rat, human, and vampire-bat tPA. Importantly, no anti-mtPA mAbs recognized muPA (Declerck et al., 1995a).

The mAbs were also assayed for their inhibitory effect on the ability of tPA to activate plasminogen (Figure 1). Interestingly, it was demonstrated that of the 21 mAbs recognizing epitopes specific for mtPA, six were inhibitory, whereas 105 of the 182 cross-reacting mAbs were inhibitory (Declerck et al., 1995a). Hence, based on the apparent correlation between inhibitory properties and cross-reactivity, it was suggested that epitopes conserved between species are functionally important for plasminogen activation (Declerck et al., 1995a). Two of the anti-mtPA mAbs were used for design of a quantitative ELISA, enabling measurements of mtPA in tissue and body fluids from mice (Declerck et al., 1995b).

Using the same overall strategy, uPA−/−mice were immunized with muPA, resulting in 38 hybridomas producing antibodies against muPA (Declerck et al., 1995a). Specificity test showed negative reaction with mtPA for all of these antibodies and interestingly, none of the anti-muPA mAbs cross-reacted with human uPA (Declerck et al., 1995a). A quantitative ELISA was designed using two of the anti-muPA mAbs (Declerck et al., 1995b). Due to the low affinity of these anti-muPA mAbs, the sensitivity of this assay was 10-fold lower than that of mtPA, precluding measurements of muPA in plasma.

In a study with the aim to generate high affinity anti-muPA mAbs with in vivo efficacy, uPA−/−mice were immunized six times, followed by three boosting injections with recombinant promuPA (Lund et al., 2008). To ensure isolation of high affinity mAbs, the wells were coated with low amounts of antigen (2 ng promuPA/well) in the hybridoma screening ELISA, resulting in identification of nine anti-muPA mAb secreting hybridomas. Especially, two mAbs (mU1 and mU3), possessing epitopes in the uPA B-chain, encompassing the catalytic site, were demonstrated to interfere with the function of mouse uPA. Specificity test demonstrated no cross-reactivity with mouse tPA, and interestingly neither...
MONOClonal antibodies AGAINST muPAR

The main function of the uPA receptor (uPAR), is to localize the proteolytic activity to the cell surface (Ellis and Dans, 1991). uPAR also interacts with vitronectin and various integrins, thereby possessing several biological functions independent of its role in uPA-mediated proteolysis (Høyer-Hansen and Lund, 2007; Smith and Marshall, 2010). Despite the multifunctional properties, uPAR+/− mice show no overt phenotype (Bugge et al., 1995).

To develop mAbs against muPAR, uPAR+/− mice were immunized with recombinant soluble muPAR (Pass et al., 2007). The procedures to generate hybridomas were identical to those described for the muPA mAbs (Pass et al., 2007; Lund et al., 2008). One fusion resulted in 12 hybridomas, secreting antibodies reacting with muPAR, including six that cross-reacted with human uPAR in Western blotting.

uPAR consists of three domains and the integrity of this structure is a prerequisite for the high affinity binding of uPA and of vitronectin (Behrendt et al., 1996; Høyer-Hansen et al., 1997). The epitopes of the anti-muPAR mAbs mR1 and mR3 are located on the N-terminal muPAR domain I, while those of mR2 and mR4 are situated on muPAR domains II-III (Pass et al., 2007; Rasch et al., 2008). They are all high affinity mAbs, with mR3 having the highest affinity (K_D = 0.5 nM), mR1, mR2, and mR4 cross-react with human uPAR and have similar affinities for uPAR from the two species (Rasch et al., 2008), mR1, mR2, and mR4 were all capable of blocking binding of the murine amino-terminal fragment of muPA (mATF) to muPAR (Figure 1; Pass et al., 2007; Rasch et al., 2008). mR1 and mR4 showed a dose-dependent inhibition of mATF binding with IC_50 values of 0.67 and 0.34 nM, respectively, being similar to that of the biological ligand (IC_50 = 0.14 nM).

Intriguingly, mR3 did not interfere with the uPA-uPAR interaction, even though the complete epitope of mR1 (Gårdsvoll et al., 2011) is close to or overlapping with that of mR3, as these mAbs are unable to bind simultaneously to muPAR (Rasch et al., 2008).

The binding site for vitronectin on uPAR is different from that for uPA (Wei et al., 1994). Some mAbs with epitopes in domain I of human uPAR block the binding of vitronectin (Høyer-Hansen et al., 1997), while none of the so far characterized mAbs, reacting with mouse uPAR domain I, prevents this interaction (Rasch et al., 2008).

The anti-muPAR mAbs were tested in rescue experiments using the above-described uPA-activatable anthrax pro-toxin assay (Pass et al., 2007). The experiments were performed both in vitro and in vivo in mice. Pre-incubation of uPAR-positive P388D.1 cells with antibody recognized human uPA. Both mU1 and mU3 are high affinity antibodies. mU3 displays higher affinity for both pro-muPA (K_D = 0.03 nM) and muPA (K_D = 0.2 nM), as compared to mU1 [pro-muPA (K_D = 0.2 nM) and muPA (K_D = 1.3 nM)]. These antibodies efficiently and dose-dependently block uPA-mediated plasminogen activation. mU1 prevents both plasmin-mediated pro-uPA activation and uPA-mediated plasminogen activation (Figure 1), while mU3 only inhibits the latter of these reactions.

To test the effect of these mAbs in vitro in a multicomponent system, a cellular assay using uPA-activatable anthrax pro-toxin (Liu et al., 2001) was employed. In this assay, a cytotoxic effect is released as a result of cell-bound uPA activity, which serves to cleave an engineered protective antigen, PrAg-U2. Pre-incubation of murine monocyte macrophage-like P388D.1 cells with either mU1 or mU3 led to significant rescue of cells simultaneously exposed to PrAg-U2 and the recombinant toxin FP59 (Lund et al., 2008). Interestingly, the rescue effect of mU1 was stronger than that of mU3 in vitro. As uPA+/− mice are insensitive to anthrax pro-toxin treatment (Liu et al., 2003), this system was used to determine the in vivo efficacy of mU1 and mU3 in wild-type mice. Two injections of the mAbs (60 mg/kg/dose) were administered to the mice prior to anthrax pro-toxin treatment. Of the wild-type mice treated with mU1, eight out of 10 mice survived, whereas only few mice treated with either mU3 or the isotype-matched negative control mAb survived, demonstrating mU1, but not mU3, to possess in vivo efficacy by blocking uPA activity (Figure 2; Lund et al., 2008, 2011a).
with mRI resulted in significant cell rescue. For the same system in vivo, it was previously demonstrated that uPAR−/− mice are insensitive to treatment with the toxins, providing evidence for the stringent need for receptor-bound uPA to release the anthrax cytotoxic effect (Liu et al., 2003). Administration of the anthrax pro-toxin simultaneously with two injections of mRI (60 mg/kg/dose) to the wild-type mice, resulted in rescue of all eight mice. Only two of seven mice treated with saline or the negative control mAb survived, thus demonstrating in vivo efficacy of mRI by blocking the uPA-uPAR binding (Pass et al., 2007).

**MONOCLONAL ANTIBODIES AGAINST mPAI-1**

PAI-1 is the primary endogenous inhibitor of both tPA and uPA. The first reported murine mAbs against mPAI-1 were generated using the same protocol as for the anti-mtPA mAbs (see above), except that the immunized mice were PAI-1−/− and mPAI-1 was the antigen (Carmeliet et al., 1993; Declerck et al., 1995b). Two fusions resulted in 64 anti-mPAI-1 mAb-producing hybridomas and two mAbs were successfully employed for design of a quantitative immunoassay for measurement of mPAI-1 in murine plasma.

In a recent study, the aim was to generate inhibitory anti-mPAI-1 mAbs reacting with the glycosylated form of mPAI-1 bound to vitronectin (Van De Craen et al., 2011). PAI-1 is secreted in an active but conformationally unstable form, which rapidly loses activity unless bound to vitronectin (Declerck et al., 1988). The desired anti-PAI-1 mAbs should therefore also inhibit PAI-1 activity of the PAI-1-vitronectin complex. Such anti-mPAI-1 mAbs were developed as previously described (Declerck et al., 1995b), though with an increase in antigen dose from 10 to 55 µg/injection. One fusion yielded 21 hybridomas producing anti-mPAI-1 antibodies. Two mAbs were specific for mPAI-1, 19 cross-reacted with rat PAI-1, and 14 of these also recognized human PAI-1. Five of the anti-mPAI-1 mAbs, reacting with epitopes conserved between the three species, were found to neutralize PAI-1 activity, even in the presence of a 33-fold molar excess of mouse vitronectin. In this neutralization assay, the inhibitory effect of the mAbs was determined as the ability of mPAI-1 to inhibit mtPA activity in the absence and presence of the antibodies (Figure 1) (Declerck et al., 1995b; Van De Craen et al., 2011).

The in vivo efficacy of the five most inhibitory mAbs was tested in a thromboplastin-dependent mouse thromboembolism model, designed to demonstrate the potential function of the mAbs to rescue injected mtPA from mPAI-1-mediated inactivation. Mice were injected with 10 mg/kg of the mAb via the tail vein. Thromboplastin was injected simultaneously with mtPA to evoke thromboembolism. A reduced physical activity was used as the read-out for the thromboembolic condition. Evaluation 15 min. after treatment, demonstrated all five inhibitory anti-mPAI-1 mAbs to possess in vivo efficacy, as the mAb-treated mice were more active than control mice (Van De Craen et al., 2011). Two of these anti-mPAI-1 mAbs have furthermore been demonstrated to decrease the level of active mPAI-1 in mouse plasma, collected from mice treated with lipopolysaccharide to induce high mPAI-1 levels in the blood (Van De Craen et al., 2012).

**MONOCLONAL ANTIBODIES AGAINST mMMPs**

**mMMP9**

Matrix metalloproteinase 9 (MMP9) belongs to the subfamily of gelatinases and is active in degradation of various components in the extracellular matrix, including early collagen cleavage fragments. Anti-mMMP9 mAbs have been generated by immunization of MMP9−/− mice with recombinant mMMP9 (Descamps et al., 2002; Opdenakker et al., 2003; Kolaczkowska et al., 2008). Hybridomas were identified by ELISA, in which the wells were coated with goat-anti-mouse IgG. Addition of hybridoma supernatants, followed by biotinylated mMMP9 was used to identify anti-mMMP9 producing clones. Of the 250 hybridomas secreting anti-MMP9 antibodies, the 16 with the strongest reactions were selected (Kolaczkowska et al., 2008). Four of these mAbs were specific for the pro-form of mMMP9, two were specific for the active form, and the 10 remaining reacted with pro-mMMP9 as well as one or more of three degradation products (Kolaczkowska et al., 2008). The mAbs reacting exclusively with the active form were found to have MMP9 neutralizing activity (Figure 1), as measured using a fluorogenic substrate (Opdenakker et al., 2003).

**mMMP14/mMT1-MMP**

All of the above-mentioned gene-deficient mice have very mild, if at all comprised phenotypes and a normal life span (Carmeliet et al., 1993, 1994, 1995; Bugge et al., 1995; Vu et al., 1998). The MMP14−/− mice have in contrast a severe phenotype and a short life span (7–13 weeks) as they develop craniofacial dysmorphism, arthritis, osteopenia, dwarfism, and fibrosis of soft tissues due to inadequate collagen turn-over (Holmbeck et al., 1999). To enable generation of anti-mMMP14 mAbs, immunizations were therefore initiated 4 weeks after birth of the MMP14−/− mice, as compared with the usual age of 8–9 weeks. The mice were only immunized twice, followed by three boosting injections with 15 µg recombinant soluble mMMP14 (Ingvarsen et al., 2008). Due to the small size of their spleens, splenocytes isolated from five immunized MMP14−/− mice were pooled for one fusion. Screening for hybridomas was performed using ELISA with each well coated with 12 mg mMMP14. Six hybridomas secreting anti-mMMP14 antibodies were isolated. The epitopes are located in the hemopexin domain and/or in the membrane-proximal linker region of mMMP14. The mAbs cross-react with the human form of MMP14. Interestingly, one mAb is capable of stimulating the MMP14 dimerization step on the cell surface, which is necessary for MMP14-mediated activation of the soluble gelatinase, MMP2. None of the mAbs possess any inhibitory effect on the collagenolytic activity of mMMP14 (Figure 1; Ingvarsen et al., 2008).

**MONOCLONAL ANTIBODIES AGAINST uPARAP/ENDO180**

uPARAP (also designated Endo180), a member of the mannose receptor family, is a collagen internalization receptor and a type I transmembrane protein (Behrendt, 2004; Engelholm et al., 2009). For the generation of mAbs, uPARAP−/− mice were immunized with recombinant soluble human uPARAP (Sulek et al., 2007). All procedures were identical to those described for generation of mAbs against muPA and muPAR (Pass et al., 2007; Lund et al., 2008). Interestingly, seven out of eight mAbs cross-react with
mouse uPARAP. The anti-uPARAP mAb 5f4 was demonstrated to inhibit cellular collagen internalization in fibroblasts (Figure 1; Madsen et al., 2011). Due to this mechanism of action, addition of 5f4 to wild-type cultured fibroblasts leads to accumulation of collagen degradation products in the cell culture medium, being similar to the collagen fragments that occur in the medium of uPARAP−/− mouse fibroblasts, cultured without antibody addition (Jürgensen et al., 2011; Madsen et al., 2011). Although the epitope of 5f4 is located to the N-terminal domains 1–3 of uPARAP (Madsen et al., 2012), the antibody does not prevent binding of uPARAP to collagen in a purified system. The capacity of blocking collagen internalization may be related to another interesting characteristic as the antibody down-regulates uPARAP expression on the cell surface (Madsen et al., 2011).

COMPARISON OF EFFECTS OF ACUTE DISRUPTION OF PROTEIN FUNCTION IN THE ADULT ANIMAL AND GENETIC DISRUPTION IN UTERO

Studies with gene-deficient animals have demonstrated uPA to play an important role in skin wound healing and extravascular fibrin clearance in the liver as well as the presence of a strong redundancy between uPA and tPA (Bugge et al., 1996). Mice deficient in uPA or tPA have a marginal delay in wound healing, while uPA−/−;tPA−/− double-deficient mice display a significant delay compared to wild-type littermates (Lund et al., 2006). To study the effect of acute disruption of uPA function in wound healing, tPA−/− mice were treated with mU1 (60 mg/kg), administered according to the mAb half-life (Jögi et al., 2010). Importantly, mU1 caused a delay in wound healing, resulting in a healing time, which was not significantly different from that of the uPA−/−;tPA−/− mice (Jögi et al., 2010). Consistent with uPA and tPA being the main plasminogen activators, a reduced level of plasmin was detected in wound extracts from mU1-treated tPA−/− mice, and the plasmin level was similar to that in uPA−/−;tPA−/− mice. This underscores that administration of mU1 blocks plasminogen activation in vivo (Jögi et al., 2010).

Both uPA−/−;tPA−/− and uPARAP−/− mice spontaneously develop hepatic fibrin deposits (Bugge et al., 1996). While uPA−/− mice occasionally display fibrin accumulation in the liver, no deposits have been detected in neither tPA−/− nor uPARAP−/− mice (Bugge et al., 1996). Hence, to evaluate the effect of mU1 and mR1 on in vivo fibrin clearance, tPA−/− mice were treated with either mAb (Jögi et al., 2007; Lund et al., 2008). Systemic treatment with mU1 as well as with mR1 resulted in hepatic fibrin accumulation in tPA−/− mice, being indistinguishable from the depositions observed in corresponding double-deficient mice (Jögi et al., 2007; Lund et al., 2008).

A recent study has demonstrated uPARAP−/− mice to possess increased accumulation of both total fibrillar collagen and collagen type IV upon CCl4-induced liver fibrosis (Madsen et al., 2012). Hepatic collagen deposition in wild-type mice with liver fibrosis thus seems as a suitable model for testing the in vivo efficacies of the anti-uPARAP mAbs.

In addition to the overlapping roles between uPA and tPA in certain physiological processes, it was recently demonstrated that ablation of MMP9 in combination with either plasminogen activator inhibitor normal gestation, resulting in a non-Mendelian distribution of the off-spring (Lund et al., 2011b). Furthermore, combined deficiency in MMP9 and uPA, but not tPA, causes a significant delay in wound healing as compared to the single-deficient and wild-type littermate control mice. Notably, a compensatory upregulation of uPA activity is seen in wound extracts from MMP9 deficient mice (Lund et al., 2011b). Consequently, these systems are interesting for studies using the described mAbs against mouse uPA and MMP9, employing wild-type or the relevant single-deficient mice for treatment.

CONCLUSION

Monoclonal antibodies (mAbs) raised in gene-deficient mice have the unique ability to recognize epitopes conserved between species. Additional reactivity with species-specific epitopes provides a more diverse reactivity with the antigen than mAbs raised in wild-type mice. Furthermore, some studies have suggested cross-reacting mAbs to be superior inhibitors to mAbs only reacting with species-specific epitopes. Compared to small molecular inhibitors, the mAbs possess an amazing selectivity, have a long half-life in circulation (3–6 days; Pass et al., 2007; Lund et al., 2008), and are therefore well-suited for therapy experiments in mouse models of different diseases, including cancer.

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