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Site-specific targeting of antibody activity *in vivo* mediated by disease-associated proteases

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Abstract

As a general strategy to selectively target antibody activity *in vivo*, a molecular architecture was designed to render binding activity dependent upon proteases in disease tissues. A protease-activated antibody (pro-antibody) targeting vascular cell adhesion molecule 1 (VCAM-1), a marker of atherosclerotic plaques, was constructed by tethering a binding site-masking peptide to the antibody via a matrix metalloprotease (MMP) susceptible linker. Pro-antibody activation *in vitro* by MMP-1 yielded a 200-fold increase in binding affinity and restored anti-VCAM-1 binding in tissue sections from ApoE(−/−) mice *ex vivo*. The pro-antibody was efficiently activated by native proteases in aorta tissue extracts from ApoE(−/−), but not from normal mice, and accumulated in aortic plaques *in vivo* with enhanced selectivity when compared to the unmodified antibody. Pro-antibody accumulation in aortic plaques was MMP-dependant, and significantly inhibited by a broad-spectrum MMP inhibitor. These results demonstrate that the activity of disease-associated proteases can be exploited to site-specifically target antibody activity *in vivo*.

Keywords

pro-antibody; protease; targeting; atherosclerosis; vascular cell adhesion protein 1

Introduction

Therapeutic antibodies are among the fastest growing classes of drugs [1, 2]. Although antibody therapeutics are generally well tolerated, they can give rise to undesirable side-effects that result from target binding in normal tissues [2, 3]. Even though such on-target antibody toxicities typically occur in small fraction of patients, they can have substantial detrimental impacts including costly failures during development [2, 4–6], post-marketing regulatory actions [3], and reduced patient compliance [7]. On-target toxicities have been reported for approved antibodies targeting epidermal growth factor receptor (skin toxicity [8]), vascular endothelial growth factor (kidney and gastrointestinal toxicity, [9]), tumor necrosis factor (infections [10]), and HER-2 (cardiotoxicity [11]). Given these problems,

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several distinct strategies have been proposed to enhance the selectivity of antibody therapeutics for diseased tissues. For example, bi-specific antibodies recognizing two distinct tumor-associated antigens have been demonstrated to increase the selectivity of targeting Erb2⁺/Erb3⁺ tumors [12]. Antibody fusions with vascular permeability enhancing agents such as IL-2 have been shown to enhance tumor uptake by several-fold [13]. The therapeutic index of Trastuzumab was improved roughly ten-fold in xenograft tumor models through co-administration of a neuropilin-1-binding peptide (iRGD), that enhanced antibody delivery into extravascular tumor tissue [14]. Despite these successes, general methods to restrict antibody binding activity to sites of disease, while sparing normal tissues, could serve to minimize on-target toxicities.

Proteolytic enzymes are known to be highly specific to, and locally activated in diverse pathologies including oncological [15], autoimmune [16], cardiovascular [17], and neurodegenerative diseases [18]. Consequently, disease-site active proteases have been exploited to locally activate cytotoxic prodrugs [19] and molecular probes for imaging tumors, arthritic joints, and atherosclerotic plaques [16, 20, 21]. In cardiovascular disease, a leading cause of death in developed countries, proteases are selectively activated in pathological atherosclerotic plaques. In particular, members of the matrix metalloprotease (MMP) family (e.g., MMP-1, 3, 8, 14) have been shown to be activated [22] and could influence plaque stability through their ability to degrade type-1 collagen in the fibrous cap [23]. Antibodies and peptide ligands specific for vascular cellular adhesion molecule-1 (VCAM-1, CD106), a marker highly expressed on plaque endothelial and smooth muscle cells [24], have proven useful for detecting plaques *in vivo* [25]. Therefore, we sought to exploit the elevated MMP activity in plaques to enhance the *in vivo* selectivity of an anti-VCAM-1 antibody. Thus, we designed a protease-activated antibody (pro-antibody) to target VCAM-1 in plaques that exhibit MMP activity. Although prodrugs face challenges with regard to their rates of local activation, we reasoned that since the serum half-life of an antibody is typically orders of magnitude greater than that of small molecule prodrugs and imaging probes, a pro-antibody might provide a more effective means to detect and respond to protease activities *in vivo*. To investigate the potential utility of pro-antibodies in enhancing *in vivo* tissue targeting selectivity, we compared the selectivity of an anti-VCAM-1 pro-antibody for targeting aortic plaques over normal tissues to that of the unmodified antibody in the widely used ApoE(−/−) mouse model [26] of atherosclerosis. ApoE (−/−) mice exhibit reduced clearance of cholesterol and triglycerides, and when fed with a high fat diet, develop atherosclerotic plaques over a period of 6–9 months that mimic many of the features of human atherosclerosis [26]. Our results demonstrate that antibody activity can be selectively targeted to pathological sites where proteases are activated, while sparing normal tissues that do not exhibit elevated protease activity.

Material and Methods

Reagents, strains, and cell lines

All experiments were performed with *E. coli* strain MC1061 (F-araD139 (ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsL (Str^R) hsdR2 (rK – mK+ mcrA mcrB1) [27] grown at 37 °C with vigorous shaking (250 rpm) in either LB medium (10 g tryptone, 5 g yeast extract, and 10 g/L NaCl) supplemented with chloramphenicol (Cm) at 34 μg/mL, or low salt LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) supplemented with 50 μg/mL Zeocin. FreeStyle 293-F (Invitrogen) cells and HEK 293 cells were grown in FreeStyle medium and DMEM with 10% FBS, respectively, supplemented with penicillin (25 units/mL), and streptomycin (12.5 μg/mL). Matrix metalloproteinase-1 (MMP-1, BIOMOL Intl.), oligonucleotides (Operon Biotechnologies, Huntsville), restriction enzymes (New England Biolabs), lipofectamine (Invitrogen), JetPEI (Genesee Scientific), protein A-agarose resin (Sigma-Aldrich), VCAM-1 (Mouse VCAM-1/Fc Chimera, R&D Systems) peroxidase-

conjugated goat anti-mouse (Jackson ImmunoResearch,), SIGMAFAST OPD (Sigma-Aldrich), DAB (3,3 Diamino Benzidine Tetrahydrochloride, 5mg tablets, MP Biomedicals), Safeguard (Fisher), Vectashield Mounting medium (Vector labs, H-1200), DPX mounting medium (Sigma) and Methyl green (Aldrich) were used without modification. Experiments were performed with the following sterile-filtered buffers: HBS-CZP buffer (10 mM HEPES, 150 mM NaCl, 2mM CaCl₂, 10 μ M ZnCl₂, 0.005% tween 20, pH 7.4), coating buffer (65 μ M Na₂CO₃, 135 μ M NaH₂CO₃) blocking buffer (PBS, 5% (w/v) BSA), dilution buffer (PBS, 0.05% (v/v) Tween 20, 0.5% (w/v) BSA), wash buffer (PBS, 0.05% (v/v) Tween 20) and TBS (20mM Tris, pH 7.4, 140 mM NaCl).

Pro-antibody construction, expression and purification

The rat anti-mouse VCAM-1 monoclonal antibody was produced using hybridoma cell line MK271 and purified with an anti-rat IgG resin [28]. A bacterial display peptide library with fifteen randomized amino acids fused to the scaffold's surface exposed *N*-terminus [29] was screened for library members that bind anti-VCAM-1 IgG. Magnetic cell separation was used to enrich anti-VCAM-1 binders (2 μ M IgG) and then binders were further screened for binding to IgG (200 nM) using FACS (fluorescence activated cell sorting), in the presence of unlabeled, pooled mouse IgG (2 μ M). The secondary biotinylated detection reagent was changed in each round (i.e., Streptavidin-Phycoerythrin (PE), Neutravidin-PE, anti-biotin IgG-PE) to disfavor peptides that bind to the secondary reagent.

To construct VCAM-1 pro-antibody expression plasmids, a scFv encoding gene was amplified by PCR, including sequence coding a GGSGGSGGS linker and MMP-1 substrate (VLVPMAMMAS) [28] on the scFv's *N*-terminus, while introducing a *Nco*I restriction site at the 3' end of the PCR product. PCR products were reamplified to add a GGSGSGG linker and anti-VCAM-1 IgG binding peptide (DGWSCVPTVLRPCA), while introducing an *Eco*RI site at the 5' end of the product. A pro-antibody with a non-binding mask that binds streptavidin (WVCHPMWEVMCLR) was similarly constructed. The resulting PCR products were digested with *Eco*RI and *Nco*I, ligated into a similarly digested pFUSE-mIgG2A-Fc2 (Invivogen), and transferred to electrocompetent *E. coli*. For antibody/pro-antibody production, HEK293 cells or FreeStyle 293-F were transfected with scFv-Fc fusion vectors. To produce a stable cell line HEK293 cells were transfected with JetPEI (20 μ g plasmid DNA / 1 x 10⁷ cells). On day 2, the medium was replaced with FreeStyle medium for expression and selection. To produce transiently transfected cells 293-F cells were transfected using Lipofectamine (375 μ g plasmid DNA / 2.5 x 10⁸ cells) and grown in FreeStyle medium. Antibody titers were measured using ELISA. ScFv-Fc was purified using protein A agarose (Pierce) following the manufacturer's protocol, concentrated using Amicon spin columns (10 kDa cutoff), and dialyzed in HBS-CZP buffer for 16 hrs.

Characterization of pro-antibody activation by ELISA and surface plasmon resonance

To assess antibody masking and protease mediated activation using ELISA the extracellular domain of VCAM-1 (R&D systems) was incubated in a 96-well plate (Nunc Immunosorb) for 12–20 hrs at 4°C (1 ng/ μ L, 100 μ L/well). Plates were blocked for 2 hrs at 37°C and antibody/pro-antibody samples were incubated either with buffer only (HBS-CZP), or with 30 nM MMP-1 for 2 hrs at 37°C. Wells were washed three times with wash buffer and incubated with the indicated dilution of antibody or pro-antibody for 2 hrs at 25°C. Wells were washed three times and incubated with peroxidase-conjugated goat-anti-mouse Fc IgG (Jackson ImmunoResearch, 0.4 nM) for 1–2 hrs at room temperature. Wells were washed three times and 200 μ L of Sigma Fast OPD solution was added to each well. Antibody binding was quantified by measurement of A_{450 nm} using a Tecan Safire spectrophotometer. Background signal (wells without antigen pre-incubation) was subtracted from the gross signal.

To confirm pro-antibody activation, binding was measured before and after treatment with MMP-1 using SPR (surface plasmon resonance, BIAcore 3000, GE Healthcare). The VCAM-1 ED was coupled to CM5 sensor chips activated with a 1:1 (v/v) mixture of 0.4 M EDC (*N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide) and 0.1 M NHS (*N*-hydroxysuccinimide) for 7 min at a flow rate of 5 μ L/min using HBS-EP running buffer. After coupling, excess activated carboxyl groups were blocked using 1 M ethanolamine (pH 8.5) injected at 5 μ L/min for 7 min. The VCAM-1 ED was diluted in 10 mM sodium acetate buffer (pH 4.5) and immobilized (2800 RU). Another flow-cell surface was activated and deactivated to serve as a reference. Binding measurements were made using HBS-CZP as the running buffer. Samples were exposed to 30 nM MMP-1 for 4 hrs at 37 °C, and both protease-treated and untreated pro-antibody were injected for 10 min at 25 μ L/min. Responses from the reference surface were subtracted.

Measurement of tissue specific protease activity 2 and ex vivo pro-antibody binding

ApoE(−/−) mice were euthanized and tissue was collected immediately following anesthesia. Samples were frozen in liquid nitrogen until used for the assay. Frozen tissue samples were homogenized in homogenizing buffer (HBS-CZP, 0.1% triton X-100 (w/v), protease inhibitor cocktail). The inhibitor cocktail (Sigma) selected did not contain MMP inhibitors or metal chelators. Homogenized samples were centrifuged at 21k rcf at 4 °C for 10 min. Supernatant was collected and frozen in nitrogen. For the assay, 5-fold dilutions of the homogenate were incubated with indicated concentrations of the antibodies for 2 hrs at 37 °C. Samples were diluted and ELISA was performed as described previously.

Purified antibody was labeled using Alexa-546 labeling kit (Invitrogen) and was tested for binding activity. For confocal microscopy imaging, anti-VCAM-1 (100 nM) antibody was used to stain explanted tissues. Samples were blocked for 1 hour with TBS supplemented with 4 % (v/v) FBS, then incubated with the antibody at 4 °C for 16 hrs, washed, and mounted with DAPI-containing mounting solution (Vector labs). For semi-quantitative analysis, samples were treated the same as above using 50 nM antibody.

Imaging of anti-VCAM-1 antibody localization after administration to ApoE(−/−) mice

Atherosclerotic plaques in transgenic mice homozygous for the Apoetm1Unc mutation (ApoE-null mice, The Jackson Laboratory) were induced by keeping the mice on high fat diet [30, 31] for 6 to 8 months. Healthy aortas were obtained from age-matched C57BL/6 wild-type mice fed on a normal diet or normal BALB/c mice. The mice were housed and all procedures performed according to standards of the UCSB Institutional Animal Care and Use Committee. To assess the *in vivo* selectivity of the anti-VCAM-1 antibody or pro-antibody for plaques, mice were injected intravenously with FITC-conjugated anti-VCAM-1 at 4 mg/kg, 80–150 μ L per injection, via the retro-orbital route under isoflurane inhalation (isoflurane 2 % –3 % (vol/vol); 2 L/min O₂). After circulation for 22 hrs, blood was cleared from anesthetized mice (under Avertin, 30 mg/mL) by perfusing with high glucose DMEM media through the left ventricle. Tissues including aorta were excised for cellular extract preparation or flash-frozen in liquid nitrogen and embedded in OCT blocks. Fresh frozen OCT-embedded tissues were serially cross-sectioned (7 μ m thickness) and immediately fixed with acetone. Samples were then blocked with Tris buffered saline (TBS) supplemented with 4% (v/v) FBS for one hour at room temperature, and then incubated with anti-FITC conjugated to peroxidase (GeneTex) diluted 1:300 in TBS supplemented with 0.4 % (v/v) FBS for 16 hrs at 4 °C. Following washing, sections were incubated with DAB (3,3'-diamino benzidine tetrahydrochloride, 5 mg tablets, MP Biomedicals) for 2–10 min., and terminated in water. Samples were stained with Methyl green (1 % (w/v), Sigma) for 4 min. Samples were dehydrated by washes with ethanol and SopV (Safeguard, Fisher) and lastly covered with DPX mounting solution (Sigma) and cover slips.

Explanted tissue sections were imaged using an Olympus Fluoview 500 microscope equipped with a 20x objective lens. An Olympus IX 70 with Q-imaging camera was used for the semi-quantitative analysis of *ex-vivo* staining (Fig. 3b) with 60x magnification. For each section, an image was acquired with blue filtration (DAPI staining), to confirm comparable cell number. An Olympus BX60 with MacroFire camera was used for imaging of immunohistochemical staining.

Measurement of protease-mediated pro-antibody binding in aortas

Explanted aorta from ApoE(+/+) and ApoE(-/-) mice were perfused with 40 mL DMEM, and cut en-face to expose plaques. Explants were incubated with FITC labeled anti-VCAM-1 pro-antibody (200 nM) or anti-VCAM FITC (200 nM; eBioscience) with or without 33 μ M Galardin (~1000 x highest known MMP IC₅₀;) in HZP buffer (minus Tween20) a slow rotary shaker (80 rpm) for 3hrs in the dark. Samples were washed (3X) with PBS at 37 °C with rotation (75 rpm) for 5 min, partitioned, and embedded in OCT for sectioning and immunohistochemical analysis.

Statistical methods

P-values indicate the confidence of statistically significant differences between samples calculated using two-tailed Student's t-test, Bonferroni's multiple comparison test, or Mann-Whitney test where indicated.

Results

Construction and characterization of an anti-VCAM-1 pro-antibody

To construct an antibody whose binding activity is activated by site specific proteolysis (i.e., pro-antibody), we reasoned that peptides that bind specifically to the antibody's antigen-binding site would effectively block or mask the antibody-antigen interaction if tethered via the *N*-terminus adjacent to the antibody binding-site (Fig. 1). Thus, binding site-masking peptide ligands were identified by screening a large bacterial display peptide library [32] using flow cytometry for binders to an anti-mouse VCAM-1 IgG. To favor high specificity for the anti-VCAM-1 antibody binding site, screening was performed in the presence of excess pooled serum IgG (2 μ M)[33]. Six cycles of sorting and re-growth enriched a population of cells displaying peptides specific for anti-VCAM-1 IgG. Three of the identified peptides (DGWSCVPTVLRPCA; RLDGWTCTVPTVEAAC; TCVPTRYGSCITDAR) exhibited a consensus of S_TCVPTVxxxC, and their apparent affinities for anti-VCAM-1 IgG were measured. The peptide with the highest apparent (bivalent) affinity (DGWSCVPTVLRPCA; K_D^{APP} = 80 nM) for anti-VCAM-1 IgG was chosen to construct a pro-antibody. The identified peptide mask was then tethered to the *N*-terminus of the antibody using a linker containing a peptide substrate (VLVPMA↓MMAS) that is efficiently hydrolyzed by collagenases including MMP-1, 8, and -14 [34]. Since this substrate, like other reported peptide substrates for MMPs, is not selective for one single MMP species, hereafter we refer to it as an MMP-cleavable substrate. For pro-antibody and antibody production, single-chain variable fragments (scFv) with and without the masking peptide were directly fused to the *N*-terminus of a mouse constant domain (Fc). Stable, antibody/pro-antibody secreting HEK293 cell lines were generated, and constructs were purified using protein A affinity chromatography (Fig. S1, Supplementary information).

Pro-antibody binding affinity is enhanced by proteolysis

To determine whether a peptide mask tethered to the antibody *N*-terminus attenuated antibody binding affinity, binding was measured before and after proteolysis. The pro-antibody exhibited a roughly 200-fold increase in apparent binding affinity after exposure to

MMP-1, when measured by ELISA (Fig. 2A). Since the unbound mask could potentially contribute to a reduced apparent binding affinity of the pro-antibody through steric blocking of the combining site or closely spaced antigen binding sites [**], we constructed a pro-antibody wherein the masking peptide was replaced with a non-binding peptide. In contrast to the masking peptide, the non-binding mask reduced affinity by less than two-fold and was unaffected by MMP-1 treatment (Fig. S2A). Similarly, surface plasmon resonance measurements revealed that the anti-VCAM-1 pro-antibody exhibited protease dependent VCAM-1 binding, while the unmodified antibody was unaffected by MMP-1 treatment (Fig. 2B, C). The pro-antibody association rate was substantially increased by proteolysis, while the dissociation rate was unchanged, indicating that the masking effect is mediated primarily by a reduction in the association rate constant.

Given the very slow association rate exhibited by the masked pro-antibody, pro-antibody association with VCAM-1 was measured over prolonged durations to simulate *in vivo* experimental conditions. Antibody and pro-antibody binding activities were measured at intervals over 24 hrs by ELISA. At low antibody concentrations (5 nM) that approximate normal tissue extravascular therapeutic IgG concentrations [35], the unmodified anti-VCAM-1 antibody exhibited maximum binding after eight hours while the pro-antibody did not exhibit detectable binding (Fig. S2B, Supplementary information). At higher concentrations (25 nM), the level of unmodified antibody binding reached equilibrium within roughly ten minutes, while the pro-antibody exhibited slow association, and an attenuated binding after four hours. As expected, pre-treatment of the pro-antibody with MMP-1 restored the normal association rate, and increased the maximum signal at equilibrium (Fig. S2C, Supplementary information). Thus, at concentrations comparable to those in normal tissues after IV administration, pro-antibody association was substantially reduced when compared to the unmodified antibody.

Plaque specific protease activity restores antibody binding activity

To determine whether pro-antibody binding to VCAM-1 was attenuated in normal tissues without MMP activity, tissue sections from ApoE(−/−) or ApoE(+/+) mice were incubated with Alexa⁵⁴⁶-conjugated antibodies or pro-antibodies, that were either pre-activated or not activated with MMP-1 (Fig. 3). The unmodified anti-VCAM-1 antibody exhibited staining in aortic plaques, and weak to moderate staining in the liver and intestine (Fig. 3). In contrast, pro-antibody staining occurred only in aortic plaques. The pro-antibody exhibited a significant reduction of staining of normal tissue ($p < 0.001$ for liver & intestine) and aortic plaque tissue sections ($p = 0.008$) Fig. 3B). However, when the same pro-antibody was pre-activated with MMP-1, staining in both normal tissues and plaques was comparable to that of the unmodified antibody. Similar results were observed in the spleen and lung tissues (Fig. S3, Supplementary information). Thus, the pro-antibody but not the antibody exhibited reduced binding in normal tissues, and pro-antibody binding could be restored by pre-activation with MMP-1.

To investigate whether MMP activity is sufficiently elevated in aortic plaques to activate pro-antibodies, activation was measured in homogenates prepared from aortas and normal tissues from both ApoE(−/−) and healthy ApoE(+/+) mice (Fig. 4). Pro-antibody binding activity resulting from exposure to tissue homogenates was measured quantitatively by ELISA. Importantly, the pro-antibody was significantly activated ($p = 0.0012$) by aortic homogenates prepared from ApoE(−/−) mice, but not those from healthy ApoE(+/+) mice (Fig. 4B). For comparison, identically prepared homogenates from the intestine did not significantly activate the anti-VCAM-1 pro-antibody. Kidney homogenates partially activated the pro-antibody at a level well below that induced by aorta homogenates. Thus, under these assay conditions, proteases present in normal tissues did not appreciably activate the pro-antibody. The VCAM-1 binding activity of the unmodified antibody was not

affected by incubation with any tissue extracts indicating that other proteases present in these samples do not modulate VCAM-1 binding activity (Fig. 4A). These results are in agreement with prior reports that MMP activity is increased in aortic plaques in the ApoE(−/−) mouse model of atherosclerosis [36], and demonstrate that plaque localized proteases can activate the anti-VCAM-1 pro-antibody.

A pro-antibody selectivity targets plaques *in vivo* in an MMP-dependent manner

To determine whether the anti-VCAM-1 pro-antibody selectively targets plaques *in vivo* in the ApoE(−/−) mouse model of atherosclerosis [26], the pro-antibody and antibody were administered by intravenous injection (4 mg/kg) to mice that were on a high fat diet for approximately 32 weeks. To assess the bio-distribution of the antibody and pro-antibody, one day after administration, tissues were recovered for immunohistochemical analysis. Analysis of frozen aorta tissue sections prepared from mice injected with the antibody or pro-antibody (n = 2) revealed large plaques that stained strongly for the presence of the anti-VCAM-1 antibody and pro-antibody (Fig. 5A, F). Antibody and pro-antibody staining in aortic plaques co-localized with the endothelial cell marker CD31 and, to a lesser extent, with macrophage cell marker CD11b (Fig. S3, Supplementary information). The unmodified anti-VCAM-1 antibody reproducibly localized at low to moderate levels in the intestine, kidney, lung and pancreas (Fig. 5B–E). In contrast, the pro-antibody was not detected in any of these tissues (Fig. 5G–J) despite the strong staining observed in plaques (Fig. 5A, F). Additionally, the pro-antibody did not localize to the aorta or other tissues of normal mice after IV administration (Fig. 5K–O). Thus, the anti-VCAM-1 pro-antibody, but not the unmodified antibody, selectively localized to aortic plaques that exhibit both VCAM-1 expression and MMP activity.

To determine whether pro-antibody accumulation in aortic plaques is mediated by proteolysis, as opposed to protease-independent binding, aorta explants were incubated with the pro-antibody in the presence and absence of the MMP inhibitor Galardin [37] (Fig. 6). In the absence of MMP inhibitor, the pro-antibody robustly stained aortic plaques from ApoE(−/−) mice but not aorta from normal mice. However, in the presence of the MMP inhibitor, pro-antibody accumulation in aortic plaques was decreased by four-fold (FITC-positive image area; p = 0.0074) and two-fold with regard to FITC fluorescence intensity (p = 0.018, n=3) (Fig. 6B). Antibody and pro-antibody staining in aortic plaques co-localized with endothelial cell marker CD31 (Fig. 6C), indicating that both molecules target activated endothelial cells. Thus, the accumulation of anti-VCAM-1 pro-antibody in aortic plaques was significantly enhanced by MMP activity present in plaques.

Discussion

Here, we demonstrated that an MMP-activated anti-VCAM-1 antibody, or pro-antibody, more selectively targets aortic plaques in ApoE(−/−) mice where MMPs are active when compared to a conventional antibody. And importantly, the pro-antibody did not accumulate to the same levels as the conventional antibody in healthy tissues at 24 hrs. Pro-antibody accumulation in aortic plaques without accumulation in healthy tissues is consistent with a localization mechanism wherein i) circulating pro-antibodies biodistribute into tissues in a manner similar to that observed for conventional antibodies, ii) locally distributed pro-antibody is activated by MMP cleavage, and iii) the activated species associates with antigen. Anti-VCAM-1 antibodies and peptides have been used previously to target imaging agents to atherosclerotic plaques [38–40] and other pathologies [41]. However, since VCAM-1 expression is up-regulated in both early and late plaques, VCAM-1 expression does not provide a measure of cardiovascular risk [42, 43]. In contrast, the local activation of MMPs capable of degrading the collagen fibrous cap [44], has been proposed to play a role in plaque destabilization [45, 46]. In agreement with prior reports, our results indicate

that MMP inhibitor sensitive proteases are activated in large advanced plaques in ApoE(-/-) mice. Thus, an MMP-activated VCAM-1 pro-antibody might be useful to differentiate plaques with low or high MMP activity. More generally, since the pro-antibody exhibited reduced accumulation in normal tissues, similarly constructed pro-antibodies could be useful for the development of *in vivo* molecular imaging and diagnostic agents.

Here, recombinant tethering of a binding-site masking peptide adjacent to the antibody binding-site of the pro-antibody provided a means to achieve high local mask concentration to drive the equilibrium pro-antibody state towards a masked state (Fig. 1). For a highly flexible linker with a time-averaged length of 1–2 nm, the local effective concentration can be estimated to be greater than 1 mM [47]. Consequently, even masks with modest affinity ($\sim 1 \mu\text{M}$) for the antibody binding-site should be capable of masking the antibody's binding activity. The reduced apparent binding affinity of the pro-antibody could be potentially be impacted by steric hinderance and/or by shielding antigen binding sites adjacent to the antigen-antibody complex as has been described for PEGylated antibodies [48]. However, a pro-antibody wherein the masking peptide did not bind to the antibody binding site exhibited only slight reductions in apparent affinity (<2 -fold) while the masked antibody exhibit more than 200-fold affinity reduction. This result indicates that the primary mechanism for affinity attenuation is through binding of the masking peptide to the antibody. In the present study, non-optimized peptide masks identified by bacterial display peptide library screening were capable of inhibiting the activity of the antibody by roughly 200-fold *in vitro*. However, *in vivo* targeting applications could benefit from an improved dynamic range between the affinity of the masked and unmasked pro-antibody. Previous studies indicate that the masking effect and dynamic range can be improved by modulating linker length and mask affinity. Covalent tethering of a masking ligand (K_D of 500 nM) to a catalytically inactive enzyme via a flexible linker increased the apparent equilibrium dissociation constant of the protein towards its ligand from 0.2 nM (without masking) to 6 μM (masked) - a 30,000-fold change in the apparent affinity [47]. Similarly, a tethered receptor ligand system consisting of tumor necrosis factor alpha (TNF α) joined to a TNF receptor fragment attenuated the activity of the fusion protein by 1000-fold [49]. These results suggest that intramolecular inhibitors of modest affinity are sufficient to attenuate intermolecular binding. From the reported study of an enzyme-tethered inhibitor [47], one can estimate that a mask affinity of 1 nM – 1 μM will maintain a balance between efficient masking, and mask dissociation upon cleavage. Given these observations, affinity maturation of masking peptides using display technologies can be applied to further attenuate pro-antibody binding activity.

Therapeutics that are selectively active at sites of pathology, or pathologically-activated therapeutics [50], represent an attractive strategy to overcome side effects that result from target blockade in normal tissues. This concept has been most extensively applied to develop small molecule prodrugs [51]. For example, the N-methyl-d-aspartate blocker Memantine, approved for the treatment of Alzheimer's disease, preferentially targets pathologically open ion channels over normal ion channels, and is well tolerated with few side effects [52]. The pathological activation of proteases in tumors has been successfully exploited to develop protease-activated cytotoxic drugs including legumain-activated doxorubicin [53], and legumain-activated auristatin [54]. Legumain-activated auristatin was capable of suppressing tumor growth and metastasis without the toxicity associated with the naked drug. In contrast to small molecule prodrugs that have short half-lives, a pro-antibody would be expected to exhibit an *in vivo* half-life comparable to an antibody (15–20 days). Although pro-antibody half-life was not measured in this study, this long half-life could provide a greatly extended window for protease-mediated activation, provided that the substrate remains stable in circulation. Consequently, similarly constructed pro-antibodies might have utility for

drugging targets present at high levels in normal tissues, that cannot be safely drugged by conventional antibodies or antibody drug conjugates.

The use of pro-antibodies to increase the selectivity of antibody targeting *in vivo* could have utility for decreasing the toxicities of antibody therapeutics, particularly those intended to act in well-defined tissue compartments including, for example, plaques, tumors and joint synovium where proteases have been shown to play critical roles in pathology. Anti-VCAM-1 therapeutic antibodies have exhibited efficacy in small animal models of arthritis [55], glioma [56], CDD-induced colitis [57], and atopic dermatitis [58]. Despite the encouraging results of these studies, successful translation of anti-VCAM-1 therapeutic antibodies may require strategies to restrict antibody activity to disease sites since a single dose of anti-VCAM-1 antibody has been shown sufficient to induce leukocytosis in healthy mice within 20 hrs [55]. agreement with this finding, antibody blockade of the VCAM-1 binding integrins has been shown to result in altered leukocyte trafficking and fetal hematopoiesis [59]. The enhanced selectivity of pro-antibodies could also be useful for increasing the selectivity of antibody-drug conjugates wherein low level targeting of normal tissues has resulted in dose limiting side effects [60, 61] and fatalities [5]. Similarly, pro-antibodies might provide a means to reduce the potential toxicities of bi-specific and multi-targeted antibodies in development [62], or to drug targets that have been discarded because of their broad tissue distribution. As has been observed for antibodies that target antigens restricted to pathological sites, pro-antibodies might be useful to avoid normal tissue sinks that increase the rate of antibody clearance from circulation [63].

Conclusions

Here, we demonstrated that a protease- activated antibody can selectively target sites of protease activation *in vivo*, while sparing normal tissues that are targeted by an antibody. Specifically, we showed that an anti-VCAM-1 pro-antibody can be activated by the atherosclerotic plaque associated protease MMP-1, and can selectively target MMP producing aortic plaques *in vivo* with reduced binding to normal and other tissues. The enhanced *in vivo* tissue selectivity of pro-antibodies may be a useful to develop diagnostic imaging agents and antibody therapeutics whose activity is restricted to sites of protease activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Protease-activated antibody (pro-antibody) design and construction. A pro-antibody is constructed by tethering a peptide (mask) that blocks the antibody's binding site to the antibody N-terminus via a flexible linker containing a protease substrate. A scFv-Fc fusion was used for simplicity. Masking attenuates binding activity until selective substrate cleavage releases the mask and fully restores antibody-binding activity.

Figure 2. Pro-antibody binding affinity is enhanced by proteolysis. (A) Binding activity of the anti-VCAM-1 pro-antibody before (○) and after (●) proteolysis, as measured by ELISA. Association and dissociation of (B) the anti-VCAM-1 antibody and (C) pro-antibody before (--) and after (-) proteolysis as measured by SPR.

Figure 3.

Analysis of anti-VCAM-1 antibody and pro-antibody binding to explanted tissue sections using fluorescence microscopy. (A) Tissue sections from ApoE(+/-) (liver, intestine) and ApoE(-/-) (aortas) mice were stained with Alexa⁵⁴⁶-conjugated anti-VCAM-1 antibody or pro-antibody (100 nM), before and after treatment with MMP-1. (Red - Alexa⁵⁴⁶; - Blue). Original magnification $\times 20$, Scale bars, 100 μ m. (B) Explanted tissues were stained with Alexa⁵⁴⁶-conjugated anti-VCAM-1 antibody or pro-antibody (50 nM). Bars represent average and standard error of the integrated fluorescence signals from 4–6 fields for each tissue. Significance was confirmed using both Student's t-test (shown), and one-way ANOVA analysis with Bonferroni's correction. However, the staining signal difference in the aorta between the pro-antibody and antibody samples did not reach statistical significance as determined using Bonferroni's multiple comparison test.

Figure 4.

Aortic plaque homogenates proteolytically activate pro-antibodies. (A) Anti-VCAM-1 Antibody and, (B) pro-antibody binding activity after incubation in assay buffer or with tissue homogenates from ApoE(-/-) or ApoE(+/-) mice, as measured by ELISA. Error bars represent the standard error of the mean of triplicate measurements. Significance was confirmed using both students T-test (shown), and one-way ANOVA analysis with Bonferroni's multiple comparison test.

Figure 5.

Immunohistochemical localization of anti-VCAM-1 antibody and pro-antibody accumulation in vivo in ApoE(-/-) and ApoE(+/+) mice. Anti-VCAM-1 antibody or pro-antibodies were administered to ApoE (-/- (A-J) or ApoE(+/+) (K-O) mice, and the indicated tissues were recovered after 24 hrs and analyzed for the presence of the antibody (A-E) or pro-antibody (F-O) by immunoperoxidase staining (indicated by brown color). Representative images (n = 4) of immunoperoxidase staining of tissues from mice injected with anti-VCAM-1 pro-antibody showed positive signal only in plaques, unlike tissues from anti-VCAM-1 antibody-treated mice. Original magnification: A,F,K: X10, B-E, G-J, L-O: X20. Scale bars, 100 μ m.

Figure 6.

Pro-antibody binding to aorta explants in the presence and absence of MMP inhibitor. (A) Cross-section of aortas comparing the bound FITC-labeled monoclonal VCAM-1 antibody and pro-antibody within luminal endothelial surface of plaque tissue (arrow; green). Original magnification $\times 20$. Scale bars, 100 μm . (B) Quantitative analysis of pro-antibody binding to plaques in the presence and absence of MMP inhibitor expressed as FITC-positive area / total selected area (Mean $\pm$ SE; from $n=3$ mice/group), and mean fluorescence intensity (Mean $\pm$ SE; from $n=3$ mice/group). (C) Localization of endothelial cell marker (anti-CD31, magenta) and anti-VCAM-1 antibody or pro-antibody-FITC (green) in aorta sections. Original magnification $\times 40$. Scale bars, 50 μm . Significance was confirmed using Student's t-test (shown) and a Mann-Whitney test.