Taylor & Francis
Taylor & Francis Group

Cancer Biology & Therapy

ISSN: 1538-4047 (Print) 1555-8576 (Online) Journal homepage: www.tandfonline.com/journals/kcbt20

Design and development of masked therapeutic antibodies to limit off-target effects: Application to anti-EGFR antibodies

Joshua M. Donaldson, Csaba Kari, Ruben C. Fragoso, Ulrich Rodeck & John C. Williams

To cite this article: Joshua M. Donaldson, Csaba Kari, Ruben C. Fragoso, Ulrich Rodeck & John C. Williams (2009) Design and development of masked therapeutic antibodies to limit off-target effects: Application to anti-EGFR antibodies, Cancer Biology & Therapy, 8:22, 2147-2152, DOI: 10.4161/cbt.8.22.9765

To link to this article: https://doi.org/10.4161/cbt.8.22.9765

Published online: 15 Nov 2009.

Submit your article to this journal

Article views: 1357

View related articles

Citing articles: 5 View citing articles

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=kcbt20

RESEARCH PAPER

Cancer Biology & Therapy 8:22, 2145-2150; 15 November, 2009; © 2009 Landes Bioscience

Design and development of masked therapeutic antibodies to limit off-target effects

Application to anti-EGFR antibodies

Joshua M. Donaldson,[1] Csaba Kari,[2] Ruben C. Fragoso,[3,†] Ulrich Rodeck[2,*] and John C. Williams[1,*]

[1]Department of Molecular Medicine; Beckman Research Institute at the City of Hope; Duarte, CA USA; [2]Department of Dermatology and Cutaneous Biology; and [3]Department of Radiation Oncology; Thomas Jefferson University; Philadelphia, PA USA

[†]Current Address: Department of Radiation Oncology; University of California Davis; Sacramento, CA USA

Keywords: monoclonal antibodies, off-target toxicity, tumor associated protease, prodrug, protein engineering, EGFR, C225-cetuximab/erbitux, 425-matuzumab

Abbreviations: EGFR, epidermal growth factor receptor; MAb, monoclonal antibody; scFv, single chain Fv fragment; CDR, complementarity determining region; MMP-9, matrix metalloprotease-9; 425, murine anti-EGFR antibody; C225, chimeric anti-EGFR antibody; SPR, surface plasmon resonance; FACS, fluorescence activated cell sorting

Therapeutic antibodies frequently cause side effects by binding antigen in non-target tissues. Here we demonstrate a novel molecular design of antibodies that addresses this problem by reversibly "masking" antibody complementarity determining regions until they reach diseased tissues containing disease-associated proteases. Specifically, two distinct single-chain Fv (scFv) fragments derived from antibodies against the epidermal growth factor receptor (cetuximab and 425) were fused a protease susceptible linker to their epitopes, which were engineered to encourage intermolecular association. Surface plasmon resonance and flow cytometry were used to confirm that the masked complex poorly interacts with native antigen, whereas protease treatment restores antigen recognition. Minimally, the "masked" scFvs possesses an eight-fold lower association with the epitope compared with the individual scFvs unmasked by proteolytic cleavage. This molecular design may have general utility for targeted release of therapeutic antibodies at disease sites.

This manuscript has been published online, prior to printing. Once the issue is complete and page numbers have been assigned, the citation will change accordingly.

Introduction

Monoclonal antibodies (mAbs) are increasingly being used in the clinical management of diverse disease states, including cancer.[1] These 'targeted agents' generally cause fewer severe side effects than traditional chemotherapy. However, adverse events have been described for many antibody therapeutics due to inadvertent antigen recognition in normal tissues. In the case of epidermal growth factor receptor (EGFR) antagonistic mAbs, dose-limiting toxicities are thought to be due to engagement of the receptor by the therapeutic antibody in normal tissues (reviewed in refs. 2 and 3).

The Erb tyrosine kinase family includes four members, of which EGFR and ErbB2/Her2 are frequently deregulated in solid tumors and are significant interest as therapeutic targets. MAbs to both antigens are used to treat various epithelial cancers. However, EGFR antagonistic mAbs, including cetuximab,[4] matuzumab,[5] and the fully human panitumumab,[6,7] can cause dose-limiting adverse events affecting primarily the skin and gastrointestinal system.[8] A similar side effect profile is observed for small molecule inhibitors of the EGFR kinase.[9]

To address this problem, we have developed and tested a new design to create antibody prodrugs (**Fig. 1**). It is based on reversible occlusion of the antigen recognition sites of mAbs through fusion with recombinant antigen fragments via a flexible linker that can be cleaved by tumor-associated proteases including MMP-9. In the tumor microenvironment, such occluded mAbs can be 'activated' by severing the linker and releasing the tethered antigenic epitope. We propose that these occluded antibodies be termed 'masked' and their activated counterparts 'unmasked.' This design was tested using two EGFR antagonistic antibodies.

Results and Discussion

Masked anti-EGFR antibody fragments were generated by cloning mutated domain III of the soluble EGFR (sEGFRdIII) N-terminus to a cleavable linker followed by single chain variable fragment (scFv) versions of the anti-EGFR antibodies matuzumab (mAb425 or 425) and cetuximab (mAbC225 or C225) (**Fig. 2A**). The mutations in each construct were designed to reduce the affinity for the attached antibody and, thus, facilitate dissociation after linker cleavage. Constructs were produced without point mutations as well. To enable proteolytic cleavage, we

*Correspondence to: Ulrich Rodeck and John C. Williams; Email: ulrich.rodeck@mail.jei.tju.edu and jwilliams@coh.org
Submitted: 06/30/09; Revised: 08/02/09; Accepted: 08/07/09
Previously published online: www.landesbioscience.com/journals/cbt/article/9765

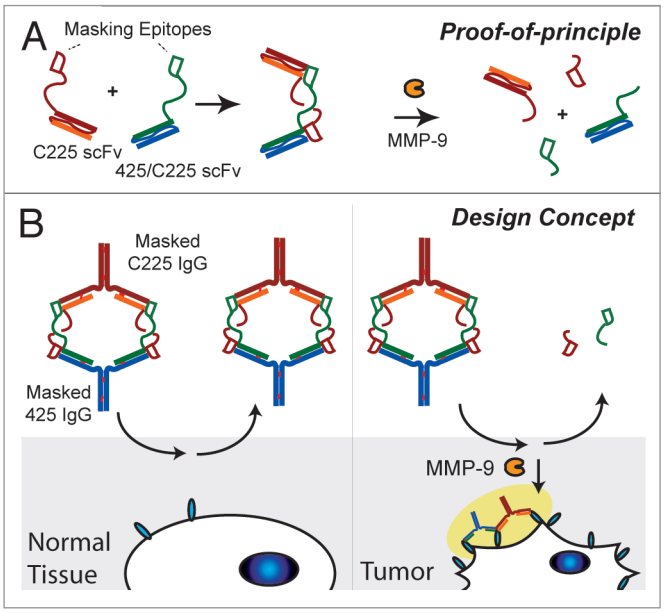

Figure 1. Antibody prodrug concept. (A) For proof-of-principle, EGFR domain III was fused to an scFv of C225 and of 425. Point mutations in EGFR domain III favor a heterodimer. In the tumor, protease cleavage is expected to release the epitope, enabling binding of the antibody to its native. (B) Schematic view of the overall design to generate IgGs that are masked and do not bind antigen in normal tissues. Cross-masking permits the simultaneous delivery of two antibodies that synergize or target two separate tumor-associated antigens.

included a metalloprotease 9 (MMP-9) substrate cleavage site in the linker, VPLSLYS.[10] MMP-9 is frequently overexpressed in epithelial malignancies in which EGFR blockade may have therapeutic benefit.[11-13]

To address potential geometric problems of epitope association with the scFv and taking into 34.7 consideration that affinity decreases as a function of linker length,[14] we designed a linker that was significantly longer than the minimal required distance. The serine-glycine rich linker consists of 12 and 19 residues flanking an MMP-9 sequence, producing an end-to-end length of approximately 133 Å. Crystal structures revealed that the distance between the C-terminus of sEGFRdIII and the N-terminus of the antibody light chains is >35.1 Å for C225,[15] and >34.7 Å for 425.[16]

The masked scFvs were produced as proteins secreted by insect cells infected with baculovirus and were purified by Ni-affinity and size exclusion chromatography (supporting data). Secreted masked mAbC225 scFv preparations linked to EGFRdIII without mutations contained a mixture of the expected length and digested fragments (data not shown). Analytical size exclusion chromatography (SEC) of the purified material indicated a dimeric molecular species, but no monomeric or oligomeric species (supporting data). Treatment of the purified material with MMP-9 and analysis by SEC indicated that the homodimeric complex was cleaved. In addition, SDS-PAGE of the protease-treated, homodimeric complex indicated that MMP-9 cleavage was specific, producing two bands of the predicted molecular weight and no cleavage of the individual domains or the scFv linker outside the intended MMP-9 site. Together, this suggests that the linker likely interferes with the intramolecular association of the epitope to the scFv (e.g., a single masked species), potentially reflecting a combination of steric clashes and an entropic penalty. These data nonetheless provide evidence that it is possible to reversibly mask the complement-determining region (CDR) of antibodies.

Since we observed a dimeric interaction, we further considered the possibility of creating a heterodimeric or crossed-masked design to simultaneously deliver two therapeutic antibodies to the tumor site. The utility of such a design is justified by recent studies in which we showed that the combination of C225 and 425 acted synergistically to inhibit EGF-stimulated cell growth and survival of breast cancer cells, and was more effective in eliciting complement-dependent tumor cell lysis than other combinations of anti-EGFR antibodies with C225.[17] To favor assembly of 'cross-masked' 425/C225 complexes, wherein the mask linked to C225 scFv binds to 425 scFv and vice versa, we altered the native epitopes by introducing point mutations that diminished intramolecular affinity but not intermolecular complex formation. Hexahistidine and FLAG tags facilitated purification and detection. Purification by affinity and SEC of the masked 425 and C225 scFvs yielded undigested material of greater than 95% purity (**Fig. 2C**). Despite the multivalent nature of the constructs, SEC did not reveal the presence of aggregates. Upon mixing, the masked 425 and masked C225 scFvs formed the expected dimeric, non-covalent 425/C225 scFv complexes, which eluted from a size exclusion column as a symmetric peak consistent with the calculated molecular mass of 106 kDa (**Fig. 2B**). As expected, addition of recombinant active MMP-9 led to complete cleavage of both antibody derivatives into the mask and scFv proteins (**Fig. 2C**). This was observed for both monomeric and dimeric molecular species.

©2009 Landes Bioscience.
Do not distribute.

The affinity of masked antibodies for immobilized sEGFRdIII was determined using surface plasmon resonance (SPR) analysis. F(ab)' fragments of the parental antibodies mAb425 and mAbC225 were used as controls to verify the immobilization and stability of the sEGFRdIII over multiple analytical cycles. The affinities of F(ab)' 425 and C225 were 91 ± 23 and 5.3 ± 0.6 nM, respectively (**Table 1**), consistent with previous work.[15] As anticipated, the scFv constructs bound with weaker affinities of 260 ± 40 and 110 ± 20 nM, respectively. We next measured antigen recognition by the masked antibodies as monomers or as a heterodimers prior to and after MMP-9 exposure. Upon mixing the CDRs of the cross-masked 425/C225 scFvs were effectively occluded, as shown by weak binding to sEGFRdIII, and treatment of the cross-masked 425/C225 complex with MMP-9 increased binding affinity by approximately an order of magnitude. Representative traces for cross-masked and individually masked scFvs are shown at 1 μM (**Fig. 3A**, blue) and 100 nM (**Fig. 3A**, yellow). Specifically, in the absence of MMP-9, the affinity for sEGFRdIII was 3.5 ± 1.1 μM, but after MMP-9 exposure, the affinity increased to 420 ± 270 nM. It is important to note that this increase represents a lower limit because the mask in this assay cannot diffuse and, therefore, competes with the immobilized antigen at a concentration equal to the antibody. As detailed below, the initial presence of the mask after MMP-9 cleavage at the tumor site should be a lesser concern. Single species masked C225 scFv and masked 425 scFv exhibited no change in affinity upon MMP-9 cleavage, consistent

with the view that the point mutations engineered into the masks or linkers effectively preclude self-association (**Fig. 3B and C**).

To measure differential association in the context of EGFR expressed on target cell surfaces, we also tested cross-masked 425/C225 antibody binding to keratinocyte-derived HaCaT cells that express EGFR. Binding was detected by flow cytometry using an AlexaFluor-labeled anti-FLAG antibody. The antibody only detected the 425 scFv because the C225 scFv lacked a FLAG tag. The cross-masked 425/C225 had low affinity for the HaCaT cells (**Fig. 4A**, red), whereas cleavage of the antibody derivatives with MMP-9 markedly enhanced their binding (**Fig. 4A**, green). Binding of the digested cross-masked 425/C225 scFvs was comparable to that of the 425 scFv alone (**Fig. 4A**, purple), indicating that the N-terminal extension remaining after digestion does not compromise binding. Finally, we noted that the masking is not absolute, as a small fraction of the antibody was bound before MMP-9 digestion as compared to the secondary alone (**Fig. 4A**, yellow). In contrast, the association of masked 425 with HaCaT cells showed little dependence on MMP-9 digestion (**Fig. 4B**). This is consistent with the SPR results and confirms that the intramolecular mask does not occlude the 425 antigen binding sites. Further studies will be required to establish that this change in affinity will be clinically relevant. However, previous research indicates that only a 3-fold change in affinity can reduce the occurrence of side effects by a third.[18] Further reducing affinity by 320-fold results in a minimum occurrence of side effects. Currently, the masking effect demonstrated here falls between these values and may be further enhanced through valency.

Collectively, these initial design strategies provide clear evidence that masking the antigen binding site and activation through a tumor-associated protease is feasible and may represent a viable means to reduce off-target effects of therapeutic mAbs. These results also highlight a path towards further improvement of biological function that will depend on masking native, bivalent antibodies. Monovalent constructs, such as Fabs and scFv, lack sufficient biological efficacy against tumor xenografts and cultured cells.[19,20] By contrast, native bivalent antibodies can not only activate immune mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)[21] but also exert anti-tumor effects by downregulating EGFR.[22] Additionally, bivalency is expected to improve the masking effect. First, overall affinity for the tumor-associated antigen is increased because of energy additivity. As a point of comparison, note that in **Figure 3B** the bivalent IgG 425 (blue) binds with much greater affinity than the monovalent scFv (purple). Secomd, in a bivalent format, the cleaved masking antigens, which are monomeric, compete poorly with tumor-derived EGFR for the unmasked antibody, as the bivalent interaction with cells should be favored over the monovalent mask interactions. Thus, the cleaved antigen is free to diffuse away from the tumor site, which favors a higher

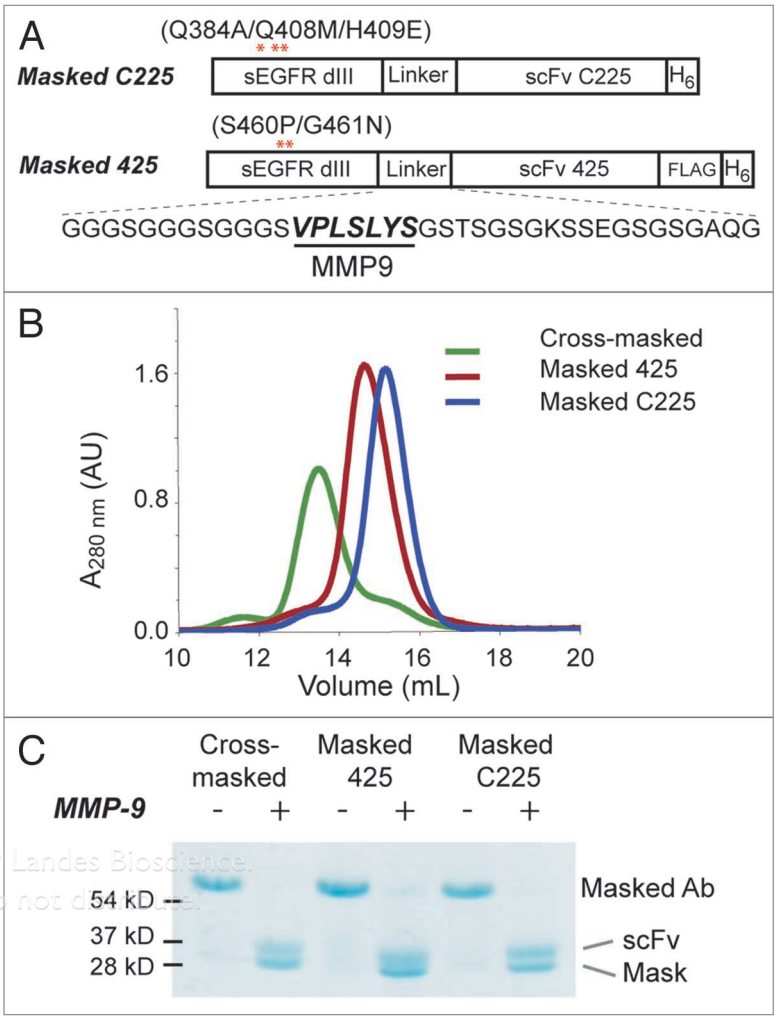

Figure 2. Design, production and characterization of cross-masked 425/C225 scFvs. (A) Topology of masked scFv constructs, indicating point mutations in EGFRdIII for either mask. The linker sequence used in both constructs is shown in bold. (B) By size exclusion chromatography, the individual masked scFvs are monomeric, whereas the admixture of C225 and 425 cross-masked scFv is consistent with a heterodimeric complex. (C) Specific cleavage of cross-masked heterodimeric scFvs and individual masked scFvs by *MMP*-9, as determined by SDS-PAGE.

Table 1. Affinity (K_D/D_{50}) of antibody fragments

Antibody fragments	Dissociation constant/D50* (nM)		Ratio
	(-) MMP-9	(+) MMP-9	
Cross-masked 425/C225	3500 ± 1100	420 ± 270	8.26 ± 5.92 †
Masked 425	240 ± 100	370 ± 110	0.66 ± 0.34
Masked C225	560 ± 800	570 ± 770	0.99 ± 1.93
scFv 425	260 ± 40		2.87 ± 0.83
Fab 425	91 ± 23		
scFv C225	110 ± 20		21.5 ± 4.7
Fab C225	5.3 ± 0.6		

*n = 3. †95% Confidence interval excludes 1

©2009 Landes Bioscience. Do not distribute.

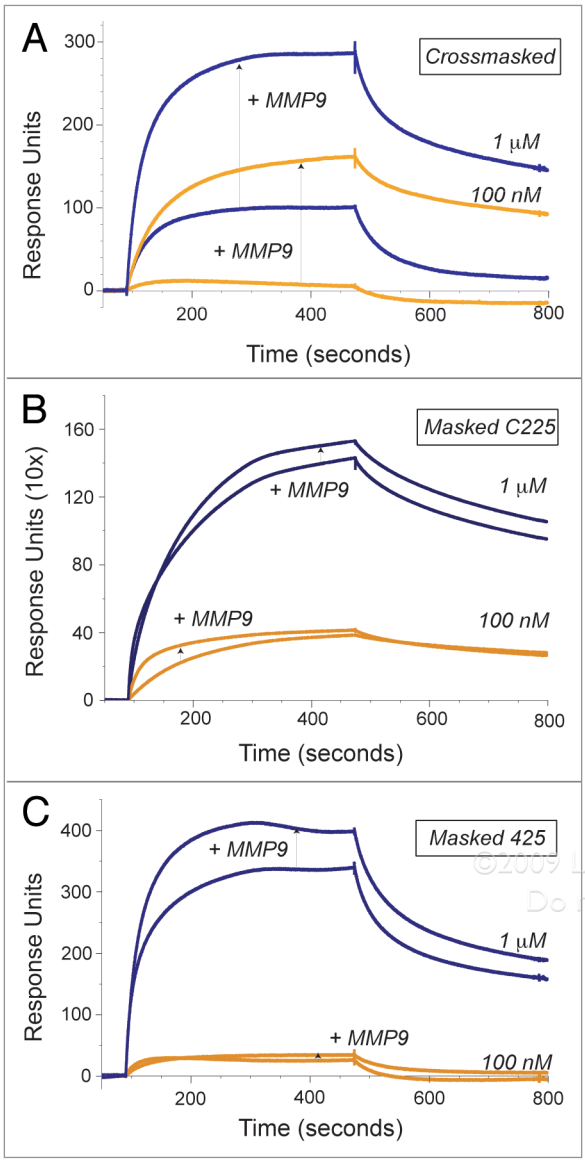

Figure 3. Binding analysis of cross-masked 425/C225 scFvs. (A) Surface plasmon resonance analysis (SPR) of cross-masked 425/C225 binding at 1 μM (blue) and 100 nM (yellow) to immobilized sEGFRdIII before and after MMP-9 digestion (indicated by arrow). (B) Control SPR of masked 425 at 1 μM (blue) and 100 nM (yellow). (C) Control SPR of masked C225 1 μM (blue) and 100 nM (yellow).

apparent affinity for the antibody than in these experiments. Third, assuming no new constraints, the occlusion of the antibody binding sites should improve (e.g., in the IgG format, the masking will be tetravalent), further reducing off-target binding. Importantly, the gain in affinity in the tetravalent format will permit the inclusion of additional mutations in the mask to further enhance the dissociation rate of the masks upon protease cleavage. We recognize that the size of the di-IgG may impede diffusion into solid tumor, yet larger protein-derived therapeutics have been used for cancer therapy in vivo. For example, therapeutic IgM and IgA antibodies have been effective in preclinical mouse models of solid tumors.[23,24] Another concern is the possibility that high molecular weight aggregates may form. However, thus far we have not observed any such aggregates, despite the potential for aggregation inherent in our current design. Finally, further improvements in the linker design, the affinities of the scFvs used, and potentially using other masking moieties such as mimetoes may all improve the design principle introduced in this report.[25] We are currently generating multivalent masked IgGs rather than scFvs to study the effects of masking on anti-tumor effects in human xenotransplants in mice.

Although these proof-of-principle design efforts were focused on EGFR mAbs, we anticipate that the design strategy will be broadly applicable to other mAbs and/or combinations used to treat cancer and chronic inflammatory processes.

Methods

Cloning and protein expression. Domain III of the soluble epidermal growth factor receptor (sEGFRdIII, comprising residues 310–512) was cloned into pAcSG2 behind the human EGFR secretion signal sequence. A flexible glycine-serine rich linker was added containing the matrix metalloproteinase 9 (MMP-9) consensus protease site (VPLSLYS) followed by a single chain variable region (scFv) anti-EGFR antibody. Point mutations were introduced into sEGFRdIII corresponding to the epitope recognized by the attached antibody. The antibody fusions were also produced without the mutations. The full linker sequence is (GGG SGG GSG GGS VPL SLY SGS TSG SGK SSE GSG SGA QG). Both scFv constructs of C225 and 425 were assembled VL-linker-VH. C-terminal FLAG (425 only) and hexahistadine tags (C225 and 425) were also added. These vectors were mixed with linearized baculovirus DNA (BD Biosciences), transfected and expanded for three rounds before confirming viral titers. Large-scale expression (5L) was conducted in suspension culture. The medium was separated from cellular material and filtered against purification buffer as described previously.[26] Protein purification was accomplished using a nickel-NTA column followed by a HiLoad 26/60 Superdex 200 preparative column (Amersham).

Assembly and digestion of antibody derivatives. Purified masked 425 [sEGFRdIII (S460P/G461N)-scFv425] and masked C225 [sEGFRdIII (Q384A/Q408M/H409E)-scFv C225] were allowed to associate for 20 min at 4°C and then loaded onto a Superdex 200 10/300 GL. Fractions corresponding to the cross-masked reagent were concentrated using a Centricon Spin Concentrator (10 kDa, Millipore). This material was then exchanged 4 times into 2 volumes of reaction buffer: 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 0.02% Nonidet P-40 substitute.[27] Active, recombinant MMP-9 (Calbiochem) was incubated with the cross-masked 425/C225 or monomeric masked scFvs at a molar ratio of 1:42. As assessed by reducing SDS-PAGE and Coomassie staining, the reaction was at least 95% complete. Samples were immediately frozen at -20°C until needed for experiments. The same digestion protocol was used to digest purified masked C225 [sEGFRdIII-scFv C225] before analysis on a Superdex 200 10/300 GL column.

SPR Analysis. Binding experiments were performed on a BIAcore T100 instrument at 25°C in HBS-EP buffer (10 mM

©2009 Landes Bioscience
Do not distribute

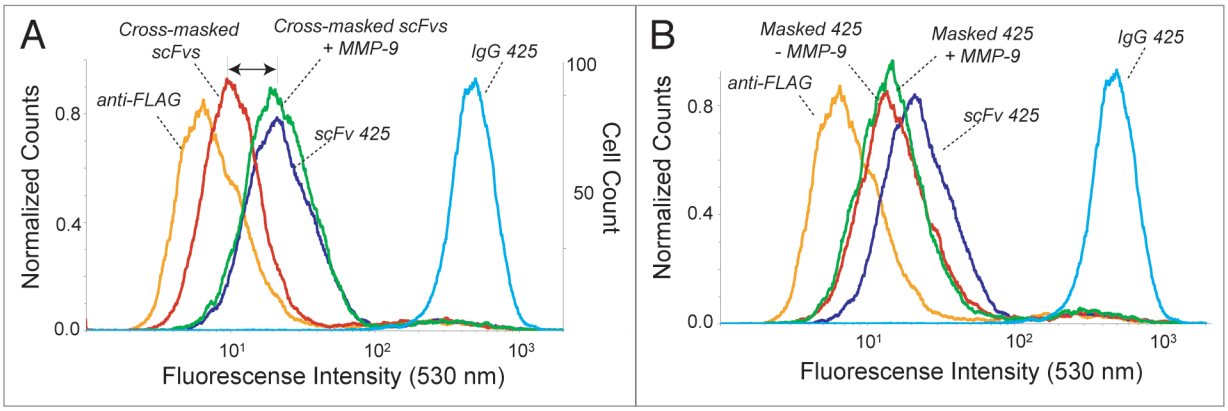

Figure 4. (A) FACS analysis of scFv binding reveals that after MMP-9 digestion the cross-masked 425/C225 (green) binds to HaCaT cells as well as the native 425 scFvs (dark blue). IgG 425 binding (light blue) confirms EGFR expression. Cell count (right axis) is for the scFv425. All others are normalized counts. (B) FACS analysis of masked 425 binding showing that binding lacks any dependence on MMP-9 activation.

HEPES, 150 mM NaCl, 3 mM EDTA, pH 8.0 and 0.005% Tween 20). Immobilization was accomplished using standard amine coupling to a CM5 chip. Blank immobilization was used for the reference cell. sEGFR domain III was applied at 50 µg/mL in acetate buffer, pH 5.5, for a target of 5,000 response units (3310 RU final). Bioactivity of the chip was confirmed by steady state binding of Fab225 at 30 µL/min. All other analyses were conducted at the same flow rate. Binding was assessed at 1 µM, 100 nM, 50 nM, 25 nM, 12.5 nM and 6.25 nM. The association phase was 380 seconds and dissociation 300 seconds. Regeneration was accomplished using 30 µL of 100 mM glycine, pH 3.0, at a flow rate of 90 µL/min. Steady state measurements were fit to the expression RU = $(R_{max} * [\ ])/([\ ] + K_d)$. Each dissociation constant was determined at least three times. The bioactivity of the chip gradually declined over time as a result of the regeneration conditions. However, minimal variation within a run permitted analysis.

FACS Analysis. Flow cytometric analyses were carried out using mAb425 and anti-FLAG (clone M2, Sigma) amine-conjugated to Alexa Fluor 488 according to the manufacturer's protocol (Molecular Probes). Between 6-8 Alexa 488 molecules were bound per antibody, as estimated by measuring the optical density at 280 nm and 494 nm. For FACS analysis, HaCaT cells were detached with trypsin/EDTA. The trypsin was inactivated with DMEM/FBS, and the cells were collected and resuspended in wash buffer (1X PBS containing 1% BSA). Approximately 1,000,000 cells were incubated at 4°C in 50 µl of digested and undigested cross-masked 425/C225, as indicated. After 45 min of incubation, cells were washed twice with wash buffer and incubated for 20 min with the anti-FLAG secondary antibody or mAb425. Samples were analyzed on a FACSCalibur (BD Biosciences). The specificity of scFv 425 binding was confirmed by pre-incubation with unlabeled mAb425 (data not shown). Also, as expected, scFv C225 binding to HaCaT cells was not detected using anti-FLAG antibodies as scFv C225 did not contain a FLAG tag (data not shown).

Acknowledgements

J.C.W. and U.R. acknowledge a Pilot Research Award from Kimmel Cancer Center, Thomas Jefferson University. U.R. acknowledges support from the American College of Radiology and Radiation Therapy Oncology Group. J.M.D. acknowledges support from the Foerderer Scholar Fellowship Award, the Dubbs Scholar Fellowship Award, the Measey MD/PhD Student Fellowship and the Ruth L. Kirschstein National Research Service Award, 5-T32-DK07705.

References

1. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. Nat Biotechnol 2005; 23:1147-57.
2. Lacouture ME, Boerner SA, Lorusso PM. Non-rash skin toxicities associated with novel targeted therapies. Clin Lung Cancer 2006; 8:36-42.
3. Rodeck U. Skin toxicity caused by EGFR antagonists-an autoinflammatory condition triggered by deregulated IL-1 signaling? J Cell Physiol 2009; 218:32-4.
4. Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res1984; 44:1002-7.
5. Rodeck U, Herlyn M, Herlyn D, Molthoff C, Atkinson B, Varello M, et al. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects. Cancer Res 1987; 47:3692-6.
6. Segaert S, Van Cutsem E. Clinical signs, pathophysiology and management of skin toxicity during therapy with epidermal growth factor receptor inhibitors. Ann Oncol 2005; 16:1425-33.
7. Van Cutsem E, Siena S, Humblet Y, Canon JL, Maurel J, Bajetta E, et al. An open-label, single-arm study assessing safety and efficacy of panitumumab in patients with metastatic colorectal cancer refractory to standard chemotherapy. Ann Oncol 2008; 19:92-8.
8. Vanhoefer U, Tewes M, Rojo F, Dirsch O, Schleucher N, Rosen O, et al. Phase I study of the humanized anti-epidermal growth factor receptor monoclonal antibody EMD72000 in patients with advanced solid tumors that express the epidermal growth factor receptor. J Clin Oncol 2004; 22:175-84.
9. Lynch TJ Jr, Kim ES, Eaby B, Garey J, West DP, Lacouture ME. Epidermal growth factor receptor inhibitor-associated cutaneous toxicities: an evolving paradigm in clinical management. Oncologist 2007; 12:610-21.
10. Turk BE, Huang LL, Piro ET, Cantley LC. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. Nat Biotechnol 2001; 19:661-7.
11. Swinson DE, Cox G, O'Byrne KJ. Coexpression of epidermal growth factor receptor with related factors is associated with a poor prognosis in non-small-cell lung cancer. Br J Cancer 2004; 91:1301-7.
12. Cox G, Jones JL, O'Byrne KJ. Matrix metalloproteinase 9 and the epidermal growth factor signal pathway in operable non-small cell lung cancer. Clin Cancer Res 2000; 6:2349-55.
13. Zhou X, Temam S, Oh M, Pungpravat N, Huang BL, Mao L, et al. Global expression-based classification of lymph node metastasis and extracapsular spread of oral tongue squamous cell carcinoma. Neoplasia 2006; 8:925-32.
14. Krishnamurthy VM, Semetey V, Bracher PJ, Shen N, Whitesides GM. Dependence of effective molarity on linker length for an intramolecular protein-ligand system. J Am Chem Soc 2007; 129:1312-20.

15. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 2005; 7:301-11.
16. Schmiedel J, Blaukat A, Li S, Knochel T, Ferguson KM. Matuzumab binding to EGFR prevents the conformational rearrangement required for dimerization. Cancer Cell 2008; 13:365-73.
17. Dechant M, Weisner W, Berger S, Peipp M, Beyer T, Schneider-Merck T, et al. Complement-dependent tumor cell lysis triggered by combinations of epidermal growth factor receptor antibodies. Cancer Res 2008; 68:4998-5003.
18. Arteaga ME, Ledon N, Casaco A, Pardo B, Garcia M, Boleda M, et al. Systemic and skin toxicity in Cercopithecus aethiops sabaeus monkeys treated during 26 weeks with a high intravenous dose of the anti-epidermal growth factor receptor monoclonal antibody Nimotuzumab. Cancer Biol Ther 2007; 6:1390-5.
19. Fan Z, Lu Y, Wu X, Mendelsohn J. Antibody-induced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells. J Biol Chem 1994; 269:27595-602.
20. Perez-Torres M, Guix M, Gonzalez A, Arteaga CL. Epidermal growth factor receptor (EGFR) antibody downregulates mutant receptors and inhibits tumors expressing EGFR mutations. J Biol Chem 2006; 281:40183-92.
21. Friedman LM, Rinon A, Schechter B, Lyass L, Lavi S, Bacus SS, et al. Synergistic downregulation of receptor tyrosine kinases by combinations of mAbs: implications for cancer immunotherapy. PNAS 2005; 102:1915-20.
22. Yan L, Hsu K, Beckman RA. Antibody-based therapy for solid tumors. Cancer J 2008; 14:178-83.
23. Azuma Y, Ishikawa Y, Kawai S, Tsunenari T, Tsunoda H, Igawa T, et al. Recombinant human hexamer-dominant IgM monoclonal antibody to ganglioside GM3 for treatment of melanoma. Clin Cancer Res 2007; 13:2745-50.
24. Nakamura K, Koike M, Shitara K, Kuwana Y, Kiuragi K, Igarashi S, et al. Chimeric anti-ganglioside GM2 antibody with antitumor activity. Cancer Res 1994; 54:1511-6.
25. Riemer AB, Kurz H, Klinger M, Scheiner O, Zielinski CC, Jensen-Jarolim E. Vaccination with cetuximab mimotopes and biological properties of induced anti-epidermal growth factor receptor antibodies. J Natl Cancer Inst 2005; 97:1663-70.
26. Ferguson KM, Darling PJ, Mohan MJ, Macatee TL, Lemmon MA. Extracellular domains drive homo- but not hetero-dimerization of erbB receptors. EMBO J 2000; 19:4632-43.
27. Backstrom JR, Lim GP, Cullen MJ, Tokes ZA. Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1-40). J Neurosci 1996; 16:7910-9.

©2009 Landes Bioscience.
Do not distribute.