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Targeting a Homogeneously Glycosylated Antibody Fc To Bind Cancer Cells Using a Synthetic Receptor Ligand

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Site-selective chemical modification of proteins has wide application in biochemistry and biotechnology, including the incorporation of labels and defined post-translational modifications onto proteins to facilitate biochemical studies and incorporation of synthetic modifications onto proteins to alter bioactivity and physical properties.[1] As part of our research into the role of N-linked glycosylation in antibody-dependent immune responses, we have needed to develop methods for targeting immunoglobulin G subclass 1 (IgG1)-type antibody fragment crystallizable (Fc) regions to bind to cancer cells in the absence of antibody fragment antigen binding (Fab) regions (Figure 1a). To accomplish this, we have developed methods to apply native chemical ligation (NCL)[2] to the site-selective modification of an expressed glycoprotein fragment. Here we report the use of these methods for the site-selective modification of a homogeneously glycosylated 225 amino acid IgG1 Fc glycoprotein with a cyclic RGD integrin antagonist as well as the results of subsequent binding and bioactivity studies.

Our strategy for targeting antibody Fc regions to bind to cancer cells is to link ligands of cell-surface receptors highly expressed on cancer cells to the N-terminus of IgG1 Fc. In this way, the ligands are displayed on the IgG1 Fc in a manner similar to how the Fab's would be displayed on a full-length antibody. Ligand binding to cellular receptors should then mimic antibody binding to antigen, displaying the Fc region in a way that should allow antibody effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) to function. As a receptor ligand to attach to IgG1 Fc, we chose the cyclic RGD peptide **1** (Figure 1b), a selective antagonist of the $\alpha_v\beta_3$ integrin receptor.[3] The $\alpha_v\beta_3$ integrin receptor is overexpressed in many types of cancer and is also involved in angiogenesis, making it a good target for delivering therapeutics to cancer cells and for imaging.[4] To achieve attachment of receptor ligands such as **1** site-selectively on the N-terminus of IgG1 Fc, we chose to use NCL because it is a chemoselective reaction that specifically modifies the N-terminus of proteins containing N-terminal cysteines, linking proteins to synthetic molecules via stable peptide bonds.[2] To use NCL for this application, it was necessary to develop methods for expressing the IgG1 Fc glycoprotein with a N-terminal cysteine and producing cyclic RGD thioester derivative **2** (Figure 1c). Also, to overcome the natural heterogeneity of glycoproteins produced in most cell lines, we utilized a glycosylation-deficient yeast strain and enzymatic digestion to produce a homogeneously glycosylated IgG1 Fc, which greatly simplified the monitoring of glycoprotein modification reactions with mass spectrometry.

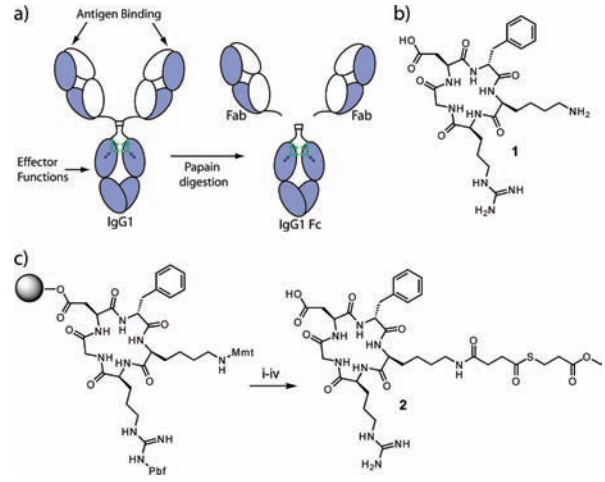

Figure 1. Antibody fragments and RGD peptides. (a) Antibody fragments obtained upon protease digestion. (b) Cyclic-RGDfK $\alpha_v\beta_3$ integrin antagonist[3] **1**. (c) Solid-phase synthesis of cyclic RGDfK thioester derivative **2**: (i) 1% TFA in CH_2Cl_2; (ii) succinic anhydride and *N*-methylmorpholine; (iii) methyl 3-mercaptopropionate, DIC, HOBt, DMAP; (iv) TFA with TIS.

NCL is a chemical reaction between N-terminal cysteines and thioesters that forms native peptide bonds.[2] Methods for expression of glycoproteins containing N-terminal cysteines have not been reported, so a method for expressing the IgG1 Fc glycoprotein with a N-terminal cysteine was developed. As a N-linked glycoprotein, IgG1 Fc must pass through the secretory pathway in order to be glycosylated, which necessitates the presence of a N-terminal signal peptide in the protein sequence to direct IgG1 Fc into the secretory pathway.[5] In addition, for efficient secretion of proteins from yeast cells, a N-terminal secretion signal, the α-factor, is required. As a result, a N-terminal cysteine-containing IgG1 Fc cannot be directly expressed but must also contain these additional peptide sequences. Since the N-terminal signal peptide and α-factor secretion signal are proteolytically removed during protein expression, we explored the possibility of using the endogenous yeast protease involved in the last step of this process, the Kex2 protease, to generate a N-terminal cysteine form of the IgG1 Fc glycoprotein. IgG1 Fc was expressed with a cysteine residue immediately following the Kex2 protease cleavage site (Figure S1 in the Supporting Information).[6] This approach worked well, and expression of this construct in *Pichia pastoris* resulted in the production of ∼30 mg/L of N-terminal cysteine-containing IgG1 Fc, designated as C-IgG1 Fc (**3**) (Figure 2a), as characterized by electrospray ionization mass spectrometry (ESI-MS) after PNGase F treatment to remove the heterogeneous N-linked

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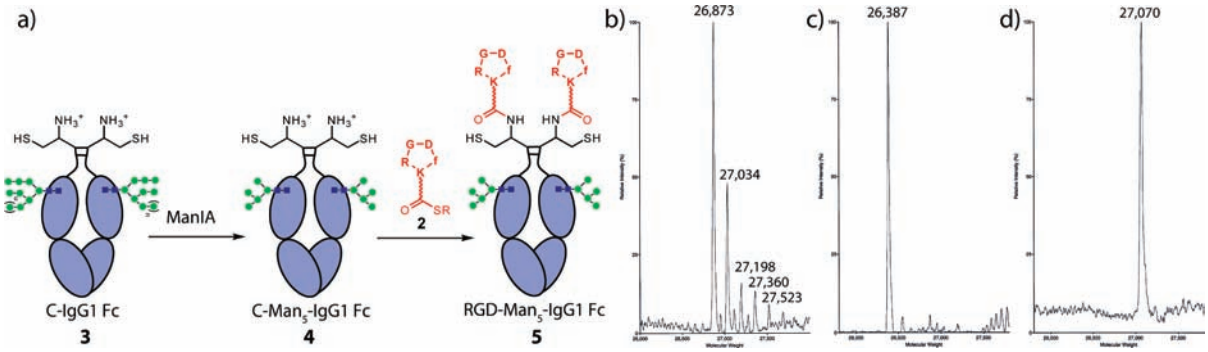

Figure 2. Synthesis of RGD-Man_5-IgG1 Fc. (a) Conversion of C-IgG1 Fc (**3**) to RGD-Man_5-IgG1 Fc (**5**) by ManIA digestion and NCL with thioester **2**. (b) ESI-MS of heterogeneously glycosylated C-IgG1 Fc (**3**). Calcd molecular weights for glycoforms containing 8−12 mannose residues: 26 872, 27 035, 27 198, 27 361, and 27 524, respectively. (c) ESI-MS of **4**: calcd, 26 386; obsd, 26 387. (d) ESI-MS of **5**: calcd, 27 071; obsd, 27 070.

oligosaccharide (Figure S3). One concern with this approach is that generation of reactive N-terminal cysteines during yeast secretory expression has the potential to form thiazolidine adducts through the reaction of the N-terminal cysteines with aldehyde- or ketone-containing metabolites.[7] Little thiazolidine formation was observed by mass spectrometry of the PNGase F-treated C-IgG1 Fc, but to ensure free N-terminal cysteines for NCL reactions, the glycoproteins used in this research were treated with hydroxylamine to hydrolyze potential thiazolidines prior to NCL reactions (Figure S3). To produce homogeneously glycosylated, N-terminal cysteine-containing IgG1 Fc, the C-IgG1 Fc glycoprotein **3** was expressed in a glycosylation-deficient yeast strain produced in our laboratory using the methods of Nett and Gerngross.[8] Expression in the glycosylation-deficient yeast strain resulted in a mixture of high-mannose C-IgG1 Fc glycoforms containing 8−12 mannoses (Figure 2b). Treatment of heterogeneously glycosylated **3** with α-1,2-mannosidase IA[9] (ManIA) to remove α-1,2 linked mannose residues produced a glycoform of C-IgG1 Fc containing five mannose residues [C-Man_5-IgG1 Fc (**4**)], with a conversion of >95% as estimated by mass spectrometry (Figure 2b,c). This homogeneous C-Man_5-IgG1 Fc **4** was then used in the NCL reactions.

The synthesis of thioester **2** was based upon the solid-phase synthesis of cyclic RGD derivatives reported by McCusker et al.,[10] except that a monomethoxytrityl (Mmt) side-chain-protected lysine was utilized (Figure 1c). After on-resin construction of the cyclic-(RGDfK) peptide, the Mmt protecting group of the lysine was selectively deprotected with 1% trifluoroacetic acid (TFA) in CH_2Cl_2; the lysine amine was then acylated with succinic anhydride. Thioester formation was effected on-resin by addition of methyl 3-mercaptopropionate, 4-dimethylaminopyridine (DMAP), and *N*-hydroxybenzotriazole (HOBt) using *N*,*N*′-diisopropylcarbodiimide (DIC) as a coupling reagent. Cleavage from the resin and removal of the remaining protecting groups were accomplished using TFA with triisopropylsilane (TIS) as a scavenger. The resulting peptide thioester **2** was precipitated from diethyl ether and purified by reversed-phase HPLC.

Ligation of C-Man_5-IgG1 Fc **4** to thioester **2** was carried out by mixing **4** (2 mg/mL, 76 μM) with 2.6 mM **2** in 20 mM sodium phosphate buffer (pH 7.5) containing 5 mM betaine and 30 mM sodium 2-mercaptoethanesulfonic acid (MESNA). The reaction mixture was incubated for 24 h, after which an aliquot of the ligation reaction mixture was analyzed by ESI-MS. As Figure 2 shows, the ligation proceeded well and was greater than 95%

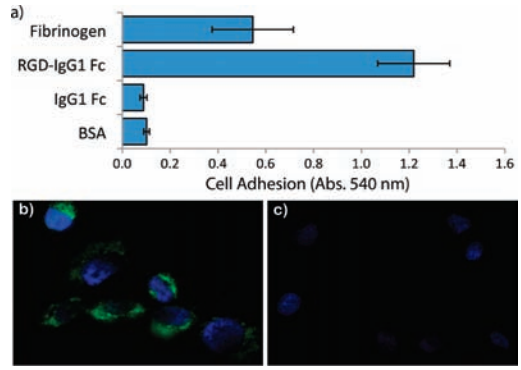

Figure 3. Cell adhesion and fluorescence microscopy. (a) WM-115 cell adhesion to microplate wells coated with 5 μg/mL of each specified protein. (b) Merged image of WM-115 cells stained with FITC-labeled RGD-Man_5-IgG1 Fc **5** and DAPI nuclear staining. (c) Merged image of WM-115 cells stained with FITC-labeled C-IgG1 Fc **3** and DAPI nuclear staining.

complete after 24 h, as estimated by ESI-MS. Site-selective modification of IgG1 Fc prevents important amino acid residues from being modified, which should improve its in vivo behavior, as has been reported for selective modification of full-length antibodies.[11] N-terminal modification of IgG1 Fc did not interfere with Fc receptor binding, as determined by ELISA binding assays (Figure S10).

To determine whether RGD-Man_5-IgG1 Fc **5** was targeted to bind $\alpha_v\beta_3$ integrin receptor-expressing cells, experiments were conducted using WM-115 melanoma cells, which express the $\alpha_v\beta_3$ integrin receptor at a high level.[12] First, an adhesion assay was conducted, taking advantage of the fact that antibodies and antibody fragments such as IgG1 Fc coat the hydrophobic surfaces of ELISA plates, allowing ELISA-type binding assays to be conducted. The ability of ELISA microplate wells coated with **5** to promote adhesion of WM-115 cells was compared with those of wells coated with fibrinogen (a natural ligand for the $\alpha_v\beta_3$ integrin receptor), C-IgG1 Fc **3**, and bovine serum albumin (BSA) (Figure 3a). Wells containing **3** alone had few cells adhering to them and were similar to the negative-control BSA wells. Interestingly, the wells coated with **5** had the highest amounts of adhering cells, ∼14-fold more than IgG1 Fc alone and also more than twice as much as adhered to the fibrinogen-coated wells (Figure 3a). This indicates strong binding interactions between RGD-Man_5-IgG1 Fc **5** on the wells and the $\alpha_v\beta_3$ integrin receptors on WM-115 cells. Additional cell lines were

also tested in this adhesion assay to confirm RGD specificity (see Figure S5).

To further assess the ability of the RGD-modified IgG1 Fc **5** to bind to the $\alpha_v\beta_3$ integrin receptor, adhesion inhibition assays comparing **5** with the free RGD peptide antagonist **1** were conducted using the methods of Wu et al.[12] Interestingly, **5** had an IC_{50} value nearly identical to that of **1** (104 and 102 nM, respectively). This indicates that the cyclic RGD modification of IgG1 Fc targets the antibody fragment to bind to $\alpha_v\beta_3$ integrin receptor-expressing cancer cells in a manner similar to the free peptide.

Fluorescence microscopy experiments were also conducted to observe the binding of the RGD-modified IgG1 Fc to WM-115 cells. Both C-IgG1 Fc **3** and RGD-IgG1 Fc **5** were labeled with fluorescein isothiocyanate (FITC) and utilized in this experiment. WM-115 cells grown on glass coverslips were incubated with 1 μM FITC-labeled **3** or **5** for 15 min at 37 °C. The cells were then washed and fixed and the cell nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI), after which the cells were mounted for fluorescence microscopy. As Figure 3 shows, there is a significant difference between the binding of FITC-labeled **5** and **3**, confirming that the RGD ligand is active for binding to the $\alpha_v\beta_3$ integrin receptor. These results were quantified (see Figure S8 and Table S2) and found to be similar to those observed for RGD dendrimers binding to the $\alpha_v\beta_3$ integrin receptor, which may indicate endocytosis of the RGD-IgG1 Fc **5**.[13]

In summary, methods to N-terminally modify glycosylated IgG1 Fc produced in yeast have been developed and applied to the N-terminal attachment of a bioactive receptor ligand, a cyclic RGD $\alpha_v\beta_3$ integrin receptor antagonist. Homogenously glycosylated C-Man_5-IgG1 Fc **4** with a N-terminal cysteine was produced by expression in a glycosylation-deficient yeast strain, utilizing Kex2 proteolytic processing to generate the N-terminal cysteine on the glycoprotein in the secretory pathway and in vitro ManIA digestion to produce the homogeneous Man_5 glycoform. A thioester-containing cyclic RGD integrin antagonist **2** was produced by solid-phase peptide synthesis and attached to the N-terminus of C-Man_5-IgG1 Fc **4** utilizing NCL. The resulting RGD-Man_5-IgG1 Fc glycoprotein **5** retained its ability to bind to and antagonize the $\alpha_v\beta_3$ integrin receptor on WM-115 melanoma cells, as demonstrated by adhesion assays, adhesion inhibition assays, and fluorescence microscopy. Antibody fragments such as IgG1 Fc have significant bioactivity in their own right, with long circulatory half-lives and the ability to direct antibody-dependent immune responses such as ADCC and CDC. Thus, attachment of cell-receptor ligands to IgG1 Fc in this manner may augment the biological activity of the attached ligands, and this possibility is under further investigation. The techniques developed here for NCL modification of expressed glycoproteins are general and may be applied to the modification of other glycoproteins or for the production of glycosylated protein fragments for the chemoenzymatic synthesis of homogeneously glycosylated glycoproteins.[14]

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Supporting Information Available: Experimental procedures and characterization data for expression of the C-IgG1 Fc **3**; synthesis of **2**, **4**, and **5**; WM-115 adhesion assay; WM-115 adhesion inhibition assay; fluorescence microscopy data; and complete ref 11b. This material is available free of charge via the Internet at http://pubs.acs.org.

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