Establishment of FUT8 Knockout Chinese Hamster Ovary Cells: An Ideal Host Cell Line for Producing Completely Defucosylated Antibodies With Enhanced Antibody-Dependent Cellular Cytotoxicity

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Abstract: To generate industrially applicable new host cell lines for antibody production with optimizing antibody-dependent cellular cytotoxicity (ADCC) we disrupted both FUT8 alleles in a Chinese hamster ovary (CHO)/DG44 cell line by sequential homologous recombination. FUT8 encodes an α-1,6-fucosyltransferase that catalyzes the transfer of fucose from GDP-fucose to N-acetylglucosamine (GlcNAc) in an α-1,6 linkage. FUT8+/−/C0/C0 cell lines have morphology and growth kinetics similar to those of the parent, and produce completely defucosylated recombinant antibodies. FUT8+/−/C0/C0-produced chimeric anti-CD20 IgG1 shows the same level of antigen-binding activity and complement-dependent cytotoxicity (CDC) as the FUT8+/+/−/C0-produced, comparable antibody, Rituxan. In contrast, FUT8+/−/C0/C0-produced anti-CD20 IgG1 strongly binds to human Fcγ-receptor IIIa (FcγRIIIa) and dramatically enhances ADCC to approximately 100-fold that of Rituxan. Our results demonstrate that FUT8+/−/C0/C0 cells are ideal host cell lines to stably produce completely defucosylated high-ADCC antibodies with fixed quality and efficacy for therapeutic use, © 2004 Wiley Periodicals, Inc.

Keywords: Chinese hamster ovary (CHO) cells; FUT8; knockout; α-1,6-fucosylation; therapeutic antibody; antibody-dependent cellular cytotoxicity (ADCC)

INTRODUCTION

Antibody-dependent cellular cytotoxicity (ADCC), a lytic attack on antibody-targeted cells, is considered one of the major immunologic mechanisms in tumor cell eradication (Cartron et al., 2002). ADCC is induced by binding of the antibody constant region (Fc) to lymphocyte receptors (FcγRs) (Clynes et al., 2000). IgG1 has two N-linked oligosaccharide chains bound to the Fc region Asn297. The N-linked oligosaccharides generally found in human serum IgG are of the complex biantennary type, composed of a trmannosyl core structure with the presence or absence of core fucose, bisecting N-acetylgalcosamine (GlcNAc) and terminal galactose, giving rise to structural heterogeneity (Rademacher et al., 1986). Recently, we demonstrated that ADCC is controlled almost solely by the absence of fucose on IgG1, and that galactose and bisecting GlcNAc contribute little or nothing to ADCC (Shinkawa et al., 2003). Shields et al. (2002) also reported that the improvement of FcγRIIIa binding to defucosylated IgG1 caused greatly enhanced ADCC (Shields et al., 2002). Thus, IgG1 defucosylation is a powerful and elegant mechanism to improve antibody effector function. However, no production system for completely defucosylated therapeutic antibodies is available.

Chinese hamster ovary (CHO) cell lines are one of the most universal hosts in biopharmaceutical production. CHO cells have several advantages for use in industry: high growth rate; high productivity; ease of genetic manipulation; good proliferation in large-scale suspension culture; and adaptability to protein-free media. A number of approved therapeutic antibodies are produced in CHO cells, including Rituxan and Herceptin, both of which are being used increasingly in the treatment of non-Hodgkin lymphoma (Leget and Czuczman, 1998) and breast cancer (Hortobagyi, 2001), respectively. However, the major form of N-linked oligosaccharides on CHO-produced antibodies are fucosylated, and the ADCC of such antibodies is much lower than the corresponding defucosylated antibodies (Shields et al., 2002; Shinkawa et al., 2003).

In mammals, almost all antibody fucose residues are attached to the innermost GlcNAc residue of N-linked oligosaccharides via an α-1,6 linkage (Miyoshi et al., 1997). α-1,6-fucosyltransferase catalyzes the transfer of fucose from GDP-fucose to the GlcNAc residue in an α-1,6 linkage in the medial Golgi cisternae (Longmore and Schachter, 1982; Wilson et al., 1976). FUT8 (Hayashi et al., 2000; Javaud et al., 2000; Uozumi et al., 1996; Yanagidani et al.,...
recombination technology. The results show that both study we report our success in the targeted disruption of event in somatic cells (Hanson and Sedivy, 1995). In this targeting, which is well known to be an extremely rare was essential. The only way to carry this out was by gene and irreversible inactivation of FUT8 function in CHO cells to efficient host cells for production of completely defucosylated antibodies.

Our goal was to generate stable host cell lines that produce completely defucosylated antibodies under commercially applicable conditions. For this purpose, complete and irreversible inactivation of FUT8 function in CHO cells was essential. The only way to carry this out was by gene targeting, which is well known to be an extremely rare event in somatic cells (Hanson and Sedivy, 1995). In this study we report our success in the targeted disruption of both FUT8 alleles in CHO/DG44 cells by homologous recombination technology. The results show that FUT8<sup>−/−</sup> cell lines express completely defucosylated recombinant IgG1s with over two orders of magnitude higher ADCC than equivalent IgG1 produced by the parent without measurable changes in antigen binding or complement-dependent cytotoxicity (CDC).

MATERIALS AND METHODS

Cell Lines

CHO/DG44 cells, in which the dihydrofolate reductase (DHFR) gene locus is deleted, were obtained from Drs. Lawrence Chasin and Gail Urlaub Chasin, Columbia University, New York (Urlaub et al., 1986).

Antibodies

Rituxan was purchased from Genentech, Inc. (South San Francisco, CA)/IDEC Pharmaceuticals (San Diego, CA). YB2/0-produced chimeric anti-CD20 IgG1 was obtained as described previously (Shinkawa et al., 2003).

Hybridoma cells producing murine anti-CCR4 monoclonal antibody KM2160 were established by immunizing mice with a peptide corresponding to 2 to 29 amino acid residues of human CCR4 (Imai et al., 1998). The heavy- and light-chain variant region cDNAs of KM2160 were cloned into the chimeric IgG1 antibody expression vector pKANTEX93 (Nakamura et al., 2000). Chimeric anti-CCR4 IgG1 was expressed in CHO/DG44 cells or YB2/0 cells and purified as described previously (Shinkawa et al., 2003).

Isolation of Chinese Hamster FUT8 cDNA

Total RNA was isolated from CHO/DG44 cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed with oligo-dT using a Superscript first-strand synthesis system for reverse transcript–polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA). A Chinese hamster FUT8 cDNA was amplified from single-stranded CHO/DG44 cell cDNAs by PCR using primers 5′-GTGTTGAAGCATAGTGGTGAC-3′ and 5′-GAGGATCGTCGATTCC-3′, designed from the murine FUT8 cDNA (Hayashi, 2000).

Targeting Construct of FUT8 Locus

The targeted disruption of the FUT8 gene in CHO/DG44 cells was carried out using two replacement vectors, pKOFUT8Neo and pKOFUT8Puro. The 9.0-kb fragment of the FUT8 gene including the first coding exon was isolated by screening the CHO-K1 cell λ genomic library (Stratagene, La Jolla, CA) with the Chinese hamster FUT8 cDNA as a probe to establish the targeting constructs. A 234-bp segment containing the translation initiation site was replaced with the neomycin-resistance gene (Neo<sup>+</sup>) cassette or the puromycin-resistance gene (Puro<sup>+</sup>) cassette from plasmid pKOSelectNeo or pKOSelectPuro (Lexicon, TX), respectively, flanked by loxP sites (Fig. 1). The diphtheria toxin gene (DT) cassette from plasmid pKOSelectDT (Lexicon) was inserted at the 5′ homologous region. The resulting targeting constructs, pKOFUT8Neo and pKOFUT8Puro, included the 1.5-kb 5′ homologous sequence and the 5.3-kb 3′ homologous sequence. Before transfection, the targeting constructs were linearized at a unique SalI site.

Transfection and Screening for Homologous Recombinants

Subconfluent CHO/DG44 cells (1.6 × 10<sup>6</sup>) were electroporated with 4 μg of linearized pKOFUT8Neo at 350 V and 250 μF using a Bio-Rad GenePulser II. After electroporation, transfectants were selected with 600 μg/mL G418 (Nacalai Tesque, Kyoto, Japan). Genomic PCR was performed in 96-well plates by the modified microextraction method reported previously (Ramirez-Solis et al., 1992) using the following primers: 5′-CTTGTGTGACTTATACTCCTCAGAG-3′ and 5′-GAGGCCACTTTGTTAGGC-3′. Homologous recombinants were identified by the 1.7-kb fragment obtained using genomic PCR and confirmed by Southern blot analysis using the 221-bp fragment amplified with the following primers: 5′-GTGA-GTCCATGGCTGTCACTG-3′ and 5′-CTTGTGTGACTTATACTCCTCAGAG-3′ (Fig. 2). The hemizygous clone was subject to a second round of homologous recombination using linearized pKOFUT8Puro and drug selection with 15 μg/mL puromycin (Sigma-Aldrich, St. Louis, MO) as described earlier. The identified homozgyous disruptants were electroporated with the Cre-recombinase expression vector pBS185 (Invitrogen) to remove drug-resistance gene cassettes from both FUT8 alleles.
Monoclonal Antibody Production by FUT8\(^{-/-}\) Cells

FUT8\(^{-/-}\) cell lines were electroporated with either the mouse/human chimeric anti-CD20 IgG1 (Shinkawa et al., 2003) or chimeric anti-CCR4 IgG1 expression vector and selected in media lacking hypoxanthine and thymidine. The confluent transfectants were cultured in Ex-Cell 301 Medium (JRH Biosciences, Lenexa, KS) for 1 week. IgG1 was purified from culture supernatants using MabSelect (Amersham Biosciences, Piscataway, NJ).

LCA Reactivity Analysis

Cells (2 × 10\(^5\)) were suspended in 50 \(\mu\)L of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and either 2 \(\mu\)g/mL fluorescein isothiocyanate (FITC)-labeled Lens culinaris agglutinin (LCA; Vector Laboratories, Burlingame, CA) or a 1/100 dilution of FITC-labeled streptavidin (KPL, Gaithersburg, MD) were added. After incubation at 4°C for 30 min, 1 × 10\(^4\) stained cells were analyzed by FACS (BD Biosciences, San Jose, CA).

IgG1 N-Linked Oligosaccharide Analysis

Fluorescence-labeled oligosaccharides were prepared from 100 \(\mu\)g of IgG1 by hydrazinolysis and pyridylamination, and analyzed by reverse-phase HPLC as described previously (Shinkawa et al., 2003). IgG1 monosaccharide composition was characterized by modified high-performance anion-exchange chromatography (HPAEC) analysis as previously reported (Shinkawa et al., 2003).

CD20 Binding Enzyme-Linked Immunoassay (ELISA)

Human B-lymphoma Raji-cell microsomal fraction (0.5 \(\mu\)g/well; JCRB CCL86) was coated onto 96-well immunoplates and incubated overnight at 4°C. Various antibody concentrations were added to the wells and incubated for 2 h at room temperature. Antibody binding to CD20 was detected by a 1/3000 dilution of goat anti-human IgG1 (H&L) peroxidase-conjugated polyclonal antibody (American Qualex, San Clemente, CA) and developed with 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt (ABTS).

Figure 1. Strategy for targeted disruption of FUT8 alleles in CHO/DG44 cells. (A) FUT8 alleles and targeting constructs. Exon 2 (■) was modified by replacing a 234-bp segment containing the translation initiation site with the drug-resistance gene cassette, flanked by two loxP sites (▲). Arrowheads represent the primers for genomic PCR. The bar indicates a probe for Southern blot analysis. After sequential targeting, the drug-resistance gene cassettes were removed from both FUT8 alleles by transient expression of Cre-recombinase. (B) Southern blot analysis of the parental, FUT8\(^{+/+}\) and FUT8\(^{-/-}\) CHO cells. Ten micrograms of genomic DNA digested with NheI was electrophoresed on a 0.6% agarose gel. (C) RT-PCR analysis of FUT8 in the parental and FUT8\(^{-/-}\) CHO cells. Single-stranded cDNAs prepared from each cell are subject to PCR using the primers to amplify full-length cDNAs of Chinese hamster FUT8.

Figure 2. LCA reactivity of CHO cells. Cells were stained with FITC-labeled LCA or FITC-labeled streptavidin and analyzed by FACS. (A) CHO/DG44 cells. (B) FUT8\(^{-/-}\) cells. (C) FUT8\(^{-/-}\) cells Ms704.
**FcγRIIlα Binding Assay**

Human Phe158 FcγRIIlα cDNA (Peltz et al., 1989) was modified by replacing the transmembrane and intracellular domains with a hexa-His tag, and then subcloned into the mammalian cell expression vector pKANTEX93 (Nakamura et al., 2000). The modified FcγRIIlα was expressed in YB2/0 cells and purified from culture supernatant using Ni-NTA chromatography (Qiagen). A 1:50 dilution of goat anti-human IgG1 (H&L) polyclonal antibody (American Qualex) was coated onto 96-well immunoplates and incubated overnight at 4°C. Sample antibodies (0.5 µg/well) were added and incubated for 1 h at room temperature. The purified FcγRIIlα–His fusion protein (0.5 µg/well) was bound to samples for 1 h at room temperature. Receptor binding was detected by a 1:1000 dilution of Penta-His conjugate (Qiagen) and developed with 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich).

**CDC Assay**

Human B-lymphoma Ramos cells (5 × 10⁴; ATCC CRL1596), 0.5 dilution of human serum complement (Sigma-Aldrich), and various antibody concentrations were incubated in 96-well flat-bottomed plates for 2 h at 37°C. WST-1 (15 µL; Roche Diagnostics, Basel, Switzerland) was added and incubated for 5 h at 37°C. Absorbance at 450 nm was measured and expressed as relative fluorescence units (RFU), reflecting viable cell density. Percent CDC was calculated according to the formula: %CDC activity = 100 × (RFU test/RFU background).

**ADCC Assay**

An anti-CCR4 antibody ADCC assay was performed by the lactate dehydrogenase (LDH) release assay using human peripheral blood mononuclear cells (PBMC) prepared from healthy donors by Lymphoprep (Axis Shield, Dundee, UK). Aliquots of target cells, murine T-cell lymphoma CCR4/El4 cells expressing human CCR4 (15), were distributed into 96-well U-bottomed plates (1 × 10⁴/50 μL) and incubated with serial dilutions of antibodies (50 μL) in the presence of human effector cells (100 μL) at an E:T ratio of 20:1. After 4-h incubation at 37°C, the supernatant LDH activity was measured using a nonradioactive cytotoxicity assay kit (Promega). Percent specific cytosis was calculated according to the formula: %specific lysis = 100 × (E – S_E – S_T)/(M – S_E) where E is the experimental release (supernatant activity from target cells incubated with antibody and effector cells), S_E is the spontaneous release in the presence of effector cells (supernatant activity from target cells incubated with effector cells), S_T is the spontaneous release of target cells (supernatant activity from target cells incubated with medium alone), and M is the maximum release of target cells (activity released from target cells lysed with 9% Triton X-100).

An ADCC assay for the anti-CD20 antibodies was performed using Raji cells and human PBMC at an E:T ratio of 20:1 as described previously (Shinkawa et al., 2003).

**RESULTS**

**Targeted Disruption of FUT8 Alleles**

Wild-type FUT8 alleles in CHO/DG44 cells were sequentially disrupted by homologous recombination using two targeting constructs (Fig. 1A). To enrich targeted clones, a positive–negative selection strategy was applied. Both targeting constructs contained the modified exon by replacing the translation initiation site with the drug-resistance gene, and the DT gene to exclude randomly integrated nonhomologous recombinants.

In the first round of gene targeting, pKOFUT8Neo was electroporated into approximately 1.0 × 10⁸ CHO/DG44 cells. Targeted insertion of the vector, reported to be common in murine embryonic stem (ES) cells (Hasty and Bradley, 1993), did not occur. However, most of the genomic PCR-positive clones were nontargeted integrants in which the introducing vector sequence had been extended by duplication of the targeted chromosomal sequence and then integrated elsewhere in the genome (Adair et al., 1989; Aratani et al., 1992; Nairn et al., 1993; Pennington and Wilson, 1991; Scheerer and Adair, 1994). Among the 41 PCR-positive clones, only 1 was identified as a homologous recombinant by Southern blot analysis. As shown in Figure 1B, the targeted convertant gave a wild-type allele-specific fragment (8.0 kb) and a recombinant allele-specific fragment (9.5 kb) at a ratio of 1:1, suggesting that there were two FUT8 loci on the targeted clone genome and that replacement occurred at one locus.

To disrupt the remaining wild-type allele, pKOFUT8-Puro was introduced into 1.6 × 10⁸ FUT8⁺/− cells. Approximately 70,000 transfectants were subjected to the screening to find that the second targeted disruption occurred in 10 clones, carrying the Neo⁺ construct on one allele and the Puro⁻ construct on the other, although >45,000 clones are required to identify only one hemizygous disruptant. In homozygous convertants, Southern blot analysis showed that a single band of the recombinant allele-specific fragment appeared and the wild-type allele-specific fragment disappeared (Fig. 1B). To exclude the drug-resistance genes from both alleles, Cre-recombinase was transiently expressed in three of the homozygous convertants, selected on the basis of good proliferation character, resulting in the generation of the FUT8⁺/− clones, Ms704, MS705, and Ms709 (Fig. 1B). All these double knockout clones were viable and exhibited morphology and growth kinetics similar to the parent.

RT-PCR analysis revealed that a mutant FUT8 transcript with deletion of approximately 200 bp was synthesized in the FUT8⁺/− clones, whereas full-length transcript was expressed in the parent (Fig. 1C), suggesting that the
translation initiation sites of both FUT8 alleles were deleted in the FUT8<sup>-/-</sup> clones.

**N-Linked Oligosaccharides in FUT8 Double Knockout Cells**

To elucidate the change of α-1,6 fucosylation on oligosaccharides by disruption of the FUT8 alleles, we analyzed the reactivity of the FUT8<sup>-/-</sup> clones against LCA, which recognizes the α-1,6-fucosylated trimannosyl-core structure of N-linked oligosaccharides. FACS analysis revealed that none of the FUT8<sup>-/-</sup> cell lines were sensitive to LCA (Fig. 2C), whereas a high concentration of LCA was bound to the parent (Fig. 2A). On the other hand, the FUT8<sup>+/−</sup> cells showed a slight change in LCA sensitivity from the parent (Fig. 2B). Thus, hemizygous disruption is not enough to reduce α-1,6 fucosylation sufficiently, and the lesion of both FUT8 alleles was necessary to completely abolish fucosylation.

Chimeric anti-CD20 IgG1 Rituxan, an approved therapeutic antibody, is commercially produced in CHO/DG44 cells (Chu and Robinson, 2001). To investigate the N-linked oligosaccharide structure of FUT8<sup>-/-</sup>-produced glycoproteins, we used chimeric anti-CD20 IgG1 expressed in FUT8<sup>-/-</sup> cell lines as a model. An anti-CD20 IgG1 expression vector, encoding the V region amino acid sequence of Rituxan, was introduced into FUT8<sup>-/-</sup> cell lines. The FUT8<sup>-/-</sup>-produced anti-CD20 IgG1 was purified from the 1-week culture supernatant in which >80% of cells had died. Typical oligosaccharide elution profiles of fluorescence-labeled FUT8<sup>-/-</sup>-produced IgG1 and Rituxan are compared in Figure 3. In Rituxan, most oligosaccharides (96%) were fucosylated and a few (4%) were defucosylated (Fig. 3A). In contrast, fucosylated oligosaccharides were not observed in FUT8<sup>-/-</sup>-produced IgG1 (Fig. 3B). The monosaccharide composition of IgG1s was analyzed to confirm the fucose content change.

FUT8<sup>-/-</sup>-produced IgG1 contained no detectable fucose residues (Fig. 4B), whereas an evident fucose peak was observed on Rituxan (Fig. 4A). In these antibodies, no difference of monosaccharide content was observed, except for fucose. We also expressed chimeric anti-CCR4 IgG1 and anti-ganglioside GD3 IgG1 in FUT8<sup>-/-</sup> cells, and detected no fucosylated oligosaccharide in either case (data not shown).

![Figure 3](image1.png)

**Figure 3.** Oligosaccharide profiles of chimeric anti-CD20 IgG1. Oligosaccharides were prepared from Rituxan (A) and FUT8<sup>-/-</sup> (Ms704)-produced IgG1 (B) and pyridylaminated to be developed by reverse-phase HPLC. The alphabetical peak codes correspond to those of oligosaccharide structure in (C). Insets show structures associated with the peaks. GN, N-acetylglucosamine; G, galactose; M, mannose; F, fucose. Peaks indicated with asterisks are artifacts of pyridylamino derivatives.

![Figure 4](image2.png)

**Figure 4.** Monosaccharide profiles of chimeric anti-CD20 IgG1. Monosaccharides, released from Rituxan (A) and FUT8<sup>-/-</sup> (Ms704)-produced IgG1 (B), were hydrolyzed and subject to HPAEC analysis. Insets show structures associated with the peaks. GlcNAc, N-acetylglucosamine; Gal, galactose; Man, mannose; F, fucose.
These findings indicated that the \(FUT8^{-/-}\) cells have no \(\alpha\)-1,6-fucosylation activity and produce completely defucosylated antibodies.

**In Vitro Activity of Defucosylated IgG1**

We examined in vitro activity of the \(FUT8^{-/-}\)-produced IgG1s to verify the effect of completely defucosylated antibodies on therapeutic efficacy. \(FUT8^{-/-}\)-produced anti-CD20 IgG1 bound CD20-positive cell membrane with an activity similar to that of Rituxan (Fig. 5A) and showed the same CDC, CD20-positive complement-mediated cell killing (Fig. 5B). Conversely, ADCC of \(FUT8^{-/-}\)-produced IgG1 was greatly enhanced compared with Rituxan (Fig. 5C). Furthermore, \(FUT8^{-/-}\)-produced IgG1 exhibited higher ADCC than rat hybridoma YB2/0-produced IgG1 (44% defucosylated), with ADCC superior to CHO-produced IgG1 (Shinkawa et al., 2003).

Next, we studied \(FUT8^{-/-}\)-produced IgG1 binding to human FcγRIIIa with Phe158, which has a lower affinity for the Fc region than that with Val158 (Koene et al., 1997). \(FUT8^{-/-}\)-produced IgG1 exhibited significantly stronger binding activity for FcγRIIIa than Rituxan, and showed higher affinity than YB2/0-produced IgG1 (Fig. 5D).

**Figure 5.** In vitro activity of chimeric anti-CD20 IgG1. (A) Raji microsomes were coated and incubated with various antibody concentrations. CD20 binding was detected by goat anti-human IgG1 polyclonal antibodies. (B) Lysis of Ramos human B lymphoma by human serum complement in the presence of various antibody concentrations was detected by WST-1. (C) Lysis of Raji human B lymphoma by human PBMC at a target:effector ratio of 1:25 in the presence of different antibody concentrations was quantified by detecting lactate dehydrogenase activity. (D) Goat anti-human IgG1 polyclonal antibodies were coated and incubated with equal amounts of sample antibodies and FcγRIIIa–His fusion protein. The binding was detected by anti-His-conjugated antibodies. (●) Rituxan; (○) CHO-produced IgG1; (△) YB2/0-produced IgG1; (○) \(FUT8^{-/-}\)-produced IgG1 (Ms704); (●) \(FUT8^{-/-}\)-produced IgG1 (Ms709).

The results of ADCC assay of chimeric anti-CCR4 IgG1 supported the preceding observations. All the \(FUT8^{-/-}\)-produced anti-CCR4 IgG1 induced greatly enhanced ADCC compared with \(FUT8^{-/-}\)-produced IgG1, with much improved FcγRIIIa binding (data not shown). These findings suggest that completely defucosylated IgG1 increases FcγRIIIa binding, thus increasing ADCC without changing either antigen binding or CDC.

**DISCUSSION**

In this study we established new host cells for the production of completely defucosylated antibodies. Recently, Shields et al. (2002) and our group (Shinkawa et al., 2003) reported the importance of IgG1 defucosylation on ADCC. Anti-HER2 IgG1 (21% fucosylated) produced by Lec13 cells, a CHO variant with reduction of GDP-fucose synthesis, showed 53-fold stronger FcγRIIIa binding than normal CHO-produced IgG1 (98% fucosylated) (Shields et al., 2002). Anti-IL-5 receptor IgG1 (34% defucosylated) and anti-CD20 IgG1 (91% defucosylated) produced by YB2/0 cells, with a low level of \(FUT8\) expression, exhibited >50-fold higher ADCC than CHO-produced IgGs (6% to 9% defucosylated) (Shinkawa et al., 2003). However, neither Lec13 cells nor YB2/0 cells produce completely defucosylated antibodies.

Gene targeting in mammalian somatic cells is difficult to achieve and requires exceedingly laborious and time-consuming processes. The difficulty is due to the fact that, in somatic cells, nonhomologous recombination occurs several orders of magnitude more frequently than homologous recombination (Sedivy and Sharp, 1989). In general, homologous recombination in somatic cells is estimated to occur with more than 100-fold lower frequency than in ES cells (Arbones et al., 1994; Hanson and Sedivy, 1995). Nevertheless, targeted disruption is the most reliable and effective way to completely remove gene function. Other tools of loss of function, for instance, antisense, Ribozyme, and RNAi, reduce, but do not eliminate, target function.

This is the first report of the establishment of CHO double knockout cell lines obtained by targeted disruption of both alleles. Although gene targeting in CHO cells has been reported previously, the target gene variation is very limited. Only four genes, \(APRT\) (Adair et al., 1989; Aratani et al., 1992; Nairn et al., 1993; Pennington and Wilson, 1991; Scheerer and Adair, 1994), \(DHFR\) (Zheng and Wilson, 1990), \(EF-2\) (Kido et al., 1991), and \(ERCC1\) (Relig et al., 1997), have been modified. In all these cases, only one targeting process was undertaken, either because hemizygous mutants were applied or the replacement of one allele was sufficient. In general, gene targeting in CHO cells is precluded without phenotypic selection strategies to accomplish high enrichment of targeted clones.

In the case of CHO cells, two additional obstacles further complicate gene targeting. First, the targeting vector frequently copies the target sequence on the CHO genome (Adair et al., 1989; Nairn et al., 1993; Pennington and
Wilson, 1991). The truncated end of the vector sequence is extended for several kilobases beyond the target homologous region on the vector (Aratani et al., 1992; Scheerer and Adair, 1994), and randomly integrated elsewhere on the genome. This event gives rise to many pseudo-homologous recombinants with the same sequence on nontarget and target loci. Such recombinants cannot be distinguished from true homologous recombinants by genomic PCR. In fact, we screened a very large number of transfectants, approximately 120,000, for targeted recombination of both FUT8 alleles, because Southern blot analysis showed almost all PCR-positive clones to be vector extension integrants. Second, chromosomal abnormalities, which significantly affect the copy number and chromosome location of the target loci (Prouty et al., 1993), have been found to accumulate in CHO cells (Siciliano et al., 1985; Warner, 1999). Most cultured somatic cell lines are aneuploid and lose native chromosome morphology because of chromosomal rearrangement during long cultivation. Assuming that several rounds of gene targeting would be necessary, we designed loxP targeting constructs in which the drug-selection gene cassettes can be removed. The clone identified with the first disruption fortunately had only two genomic FUT8 gene copies (Fig. 1B). Thus, only two rounds of targeting were required to inactivate FUT8 gene function.

Hemizygous disruption of the FUT8 allele is not sufficient to reduce α-1,6 fucosylation. FUT8+/− cells showed a slight change in LCA sensitivity compared with the parent (Fig. 2B). FUT8+/−-produced anti-CCR4 IgG1 exhibited few changes in fucosylation and a slight ADCC improvement (data not shown). The finding that α-1,6-fucosyltransferase activity does not correlate with FUT8 gene dosage in CHO cells may be interpreted two ways. One possibility is that expression of FUT8 from only one allele is phenotypically sufficient in CHO cells. The other is that the FUT8 gene is a "functional hemizygous" (Siminovitch, 1985); the FUT8 alleles on the CHO genome may differentially contribute to the phenotype, or either allele may be inactivated. To clarify which mechanism is correct, further studies are required.

FUT8 is the sole gene responsible for α-1,6-fucosyltransferase activity in CHO cells. In FUT8−/− cells, surface α-1,6 fucosylation disappeared (Fig. 2C), and recombinant IgG1s were completely defucosylated (Figs. 3 and 4), suggesting loss of all α-1,6-fucosyltransferase activity. FUT8 is the only successfully cloned mammalian gene encoding an α-1,6-fucosyltransferase (Uozumi et al., 1996; Yanagidani et al., 1997), although several studies have assumed the existence of other α-1,6-fucosyltransferase gene families (Kaminska et al., 1998; Miyoshi et al., 1997; Struppe and Staudacher, 2000). Our results suggest that the FUT8 gene is not redundant in mammals.

ADCC of FUT8−/−-produced IgG1s was improved by more than two orders of magnitude compared with FUT8+/−-produced IgG1s. FUT8−/−-produced anti-CD20 IgG1 and anti-CCRF4 IgG1 exhibited >100-fold (Fig. 5C) and 1000-fold (data not shown) higher ADCC, respectively, without a change in antigen-binding activity. The differing improvement in ADCC between the two antibodies may be due to cell lysis efficiency closely related to the type of antigen. Moreover, FUT8+/−-produced IgG1 (100% defucosylated) increased ADCC much more than YB2/0-produced IgG1 (44% defucosylated). FUT8−/−-produced IgG1s bound to FcγRIIIa with significantly more strength than YB2/0-produced IgG1, suggesting that the affinity of FcγRIIIa for the Fc region is directly related to IgG1 defucosylation (Fig. 5D). The mechanism for improvement of FcγRIIIa binding with antibody defucosylation is still an open question. There is no specific interaction between fucose and other amino acid residues on the Fc (Krapp et al., 2003). In the crystal structure of the FcγRIIIb–IgG1 Fc complex, the fucose residue has been shown to have no contact with the receptor (Radakovits et al., 2001). However, our study using completely defucosylated antibodies has provided direct evidence that defucosylation plays a critical role in the improvement of FcγRIIIa binding to greatly elevate ADCC, strongly suggesting that antibody defucosylation could contribute to conformational change of the CH2 region, attached to the oligosaccharides, to enhance FcγRIIIa binding.

FUT8−/−-produced anti-CD20 IgG1 shows the same CDC as Rituxan (Fig. 5B), demonstrating that complete defucosylation does not affect CDC. CDC, a complement-mediated lysis of target cells, is one of the major effector functions of antibodies. It is induced by two types of complement activation cascades, the classical or alternative pathways, both of which are influenced by N-linked oligosaccharide structure of the Fc region (Boyd et al., 1995; Jefferis et al., 1998; Tsuchiya et al., 1989). A previous study using Lec13-produced IgG1 reported that fucose did not contribute toClr binding, the first step in the classical CDC pathway (Shields et al., 2002). We confirmed that IgG1 defucosylation does not reduce CDC. Elimination of α-1,6-fucosylation activity does not affect cell viability, growth, and protein productivity. FUT8 is highly expressed in various human cancer cell lines (Miyoshi et al., 1997). The level of α-1,6-fucosylation in N-linked oligosaccharides is known to be elevated along with carcinogenesis in mammalian liver and ovary (Noda et al., 1998a, 1998b; Takahashi et al., 2000). Hence, we had concern about the impact of disruption of the FUT8 alleles on proliferation of CHO cells in which a high level of FUT8 expression is observed (Miyoshi et al., 1999; Shinkawa et al., 2003). Contrary to our concerns, FUT8−/− cells are viable and have growth kinetics and morphologies similar to the parent CHO/DG44 cells. We expressed several types of IgG1s in FUT8−/− cells and found that all antibodies have normal antigen binding and enhanced ADCC, and that there is no observable difference in productivity from the parent. In addition, we carried out fed-batch suspension cultures using these cell lines and observed no change of defucosylation, with specific productivity rate comparable to that of the parent (data
new host cells for production of completely defucosylated antibodies. A cell line is expected to achieve both improved efficacy and reduction of dose and cost. Moreover, \( FUT8^{+/+} \) cell lines have the great advantage of providing uniformity to biopharmaceutical N-linked glycosylation. \( FUT8^{+/+} \) CHO/DG44 cell lines, the ideal host cells to stably produce high-ADCC therapeutic antibodies, are now available.

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References


Reference


