Correction of cross-linker sensitivity of Fanconi anemia group F cells by CD33-mediated protein transfer

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Introduction

The role of gene transfer into hematopoietic cells for the eventual goal of gene therapy of hematopoietic disorders is firmly established, albeit challenging.1,2 By contrast, the introduction of proteins3,4 directly into hematopoietic cells as a potential therapeutic strategy has received less attention. This potentially powerful strategy can be envisaged to provide either definitive or adjunctive therapy for hematopoietic disorders, but the choice of appropriate therapeutic peptides and models of hematopoietic disorders will be critical in the evaluation of this paradigm. The autosomal recessive disorder Fanconi anemia (FA) fulfills many of the criteria needed for the study of protein transfer. FA is an important model of bone marrow failure, and death in FA usually results from complications of hematopoietic failure.5,6 Therefore, the rescue of hematopoietic cells in this disorder could significantly decrease the morbidity and mortality of FA patients. The hypersensitivity of FA cells to bifunctional alkylating agents, such as mitomycin C (MMC), also provides a straightforward assay for the evaluation of potentially therapeutic proteins. This assay has formed the cornerstone of the functional cloning experiments of all 6 FA disease genes cloned to date, including those for complementation groups C (FANCC),7 A (FANCA),8 G (FANCG),9 F (FANCF),10 E (FANCE),11 and D2 (FANCD2).12 Relevant to this study, the FANCF gene encodes a 374-amino acid product with no homology to other proteins, but of hematopoietic failure. Therefore, the rescue of hematopoietic marrow failure, and death in FA usually results from complications for the study of protein transfer. FA is an important model of bone therapy for hematopoietic disorders, but the choice of appropriate strategy can be envisaged to provide either definitive or adjunctive strategy has received less attention. This potentially powerful approach by using an antibody single-chain fusion protein to correct the phenotypic defect in FA group F cells. A 68.5-kd chimeric protein (His-M195FANCF) was expressed, consisting of a His tag, a single-chain antibody to the myeloid antigen CD33, and the FANCF protein, as well as a 43-kd His-FANCF fusion protein lacking the antibody motif, in Escherichia coli. The nickel-agarose--purified His-M195FANCF protein bound specifically to the surface of HeLa cells transfected with CD33 and internalized through vesicular structures. The fusion protein, but not CD33, sorted to the nucleus, consistent with the known nuclear localization of FANCF. No similar binding or internalization was observed with His-FANCF. Pretreatment of the transfected cells with chloroquine abolished nuclear accumulation, but there was little change with brefeldin A, indicating a minimal role for the Golgi apparatus in mediating transport from endosomes to the cytosol and the nucleus. The intracellular half-life of His-M195FANCF was approximately 160 minutes. Treatment of CD33-transfected FA group F lymphoblastoid cells with 0.1 mg/mL His-M195FANCF conferred resistance to mitomycin C. No similar protection was noted in CD33 parental cells or CD33 FA cells belonging to groups A and C. These results demonstrate that antibody-directed, receptor-mediated protein transfer is a versatile method for the delivery of biologically active proteins into hematopoietic cells. (Blood. 2001;98:3817-3822)

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Here we evaluated the versatility of another receptor-mediated approach for the transfer of a bioactive protein into FA group F cells. CD33 is a transmembrane glycoprotein that is expressed primarily on normal myeloid progenitor cells as well as on about 80% of acute myelogenous leukemia (AML) cells. Although the physiologic function of CD33 is unclear and a ligand has not yet been identified, the known ability of CD33 to undergo endocytosis upon binding a monoclonal antibody has been exploited to target AML cells with monoclonal antibodies conjugated to cytotoxic drugs. Here we describe the construction of a novel single-chain antigen-binding protein from such a monoclonal antibody and show that a fusion protein consisting of the antibody and the FANCF protein can be targeted to FA group F cells expressing CD33, resulting in correction of the major phenotypic defect of these cells. We suggest that this particular approach or similar ones may be of use in the therapy of selected hematopoietic disorders.

Materials and methods

Constructs

To generate a single-chain anti-CD33 antibody cassette, we obtained the humanized variable L-chain (V_L) and H-chain (V_H) cDNAs (HuMI95-pVk and HuMI95-pVg, respectively) of MI95, a murine monoclonal antibody that binds to CD33 (Dr C. Queen, Protein Design Labs, Fremont, CA). A 16–amino acid linker was inserted between the V_L and V_H chains by overlapping polymerase chain reaction (PCR) fragments. An Ascl site was interposed in the middle of the linker to facilitate subcloning. The following PCR primer pairs and templates were used to derive 2 PCR fragments overlapping at an AscI restriction site.

The V_L-linker fragment was derived in 2 steps. V_L-Bam (5'-GGGGATCCGCAATTAGCTAGGGCCGCGCACGCT-3') is the forward primer spanning from the 5'-end of V_L, Link1-V_L (5'-AGAGGATGAACTTTGTGATCCTCACCCTTGGT-3') is the reverse primer spanning the 5'-end of the linker and the 3'-end of V_L. The template used was the vector HuMI95-pVk, and the resulting fragment is called PCR-A. The 3'-end of the linker was extended using V_L-Bam and Link2-V_L (5'-GGGGGCCCAGAACCAGAGGTA-GAACCTTTT-3') is a reverse primer from the middle of the linker and overlapping partially with Link1-V_L. PCR-A was used as the template to yield PCR-B. The latter fragment was flanked by the unique restriction sites BamHI at the 5'-end and Ascl at the 3'-end and contained the entire coding sequence of MI95 V_L followed by approximately half the coding sequence of the linker.

The linker-V_H fragment was derived using a similar 2-step strategy. Link1-V_H (5'-GGAGGTAAAGGTCAGGTTCAGCTGGTGCA-3') is a forward primer from the 3'-end of the linker and extending into the 5'-end of V_H, and PE-Not (5'-GGGAATTCGCGGCCTAGAGACACGTTA-3') is a reverse primer encoding the 3'-end of V_H preceded by a NotI site. The vector HuMI95-pVg was used as template to yield the fragment PCR-C. The latter was extended at the 5'-end using primers Link2-V_H (5'-GGGGGCCCAGAACCAGGTA-GAACCTTTT-3') and Link3-V_H (5'-GGGGGCCCAGAACCAGGTA-GAACCTTTT-3'). The former was ligated with the latter to yield the complete fusion cDNA, encoding the midportion of the linker preceded by an Ascl site, and PE-Not to yield PCR-D.

PCR-B was cleaved with BamHI and Ascl, and PCR-D with Ascl and NotI. Both fragments were cloned in a 3-way ligation at the BamHI and NotI sites of pBluescript KS (Stratagene, La Jolla, CA). The entire fusion cDNA (herein called MI95) was sequenced to confirm that the desired changes had occurred.

To generate prokaryotic expression constructs, we modified the full-length human FANCF cDNA at the 5'-terminus by introduction of a NotI site by PCR. The M195 cDNA and the modified FANCF cDNA were cloned into the corresponding restriction sites of a hexahistidine (Hex)—encoding pQE vector (Qiagen, Santa Clarita, CA) to obtain pQE9-M195FANCF. Using a similar strategy, we also cloned full-length FANCF without M195 in pQE9. A PstI fragment encoding FANCF residues 5 to 175 was also cloned in pQE31. Full-length human CD33 (gift of Dr B. Seed) was cloned in the episomal expression vector pREP4 for transfection of lymphoblastoid cells and in pcDNA3 for transfection of HeLa and 293 cells.

Antibodies

A commercially available mouse monoclonal antibody to CD33 (CalTag Laboratories, Burlingame, CA) was used at a 1:50 dilution in immunofluorescence experiments. Polyclonal antibodies to residues 5 to 175 of FANCF were generated by immunizing rabbits against the corresponding His-tagged protein expressed in Escherichia coli, as before. Secondary antibodies included fluorescein isothiocyanate (FITC)—conjugated goat anti–rabbit IgG and Texas red–conjugated goat anti–mouse IgG (Molecular Probes, Eugene, OR). The same CD33 antibody was also used in some experiments for fluorescence-activated cell sorting (FACS).

Cell culture and transfection

FA lymphoblastoid cells were cultured in RPMI 1640 medium and 10% fetal calf serum (FCS) and supplemented with 1 mM sodium pyruvate and 2 mM L-glutamine. Stably transfected lymphoblasts generated by electroporation were maintained in the same medium in the presence of 200 μg/mL hygromycin B. All FA cell lines have been described previously. The EUFA698 cell line is homozygous for the 230-252del mutation in FANCF. The HSC536 (FA group C) and HSC72 (FA group A) lymphoblastoid cells have been described previously. HeLa cells and 293 cells were maintained in RPMI 1640 with 10% FCS. Transfection of these cells with pcDNA-based vectors was done by lipofection, as described. Both transiently and stably transfected HeLa cells were used. Stably transfected HeLa cells were obtained by selection in G418, isolation and expansion of individual clones, and confirmation of surface expression for CD33 by FACS analysis.

Expression and purification of His-tagged proteins

His-tagged protein used for the protein transduction studies was prepared by a modification of a method previously shown to yield correctly folded proteins from inclusion bodies. E coli M15 [pREP4] (Qiagen) transformed with pQE9-based vectors was cultured overnight at 37°C in 50 mL of Luria broth in the presence of ampicillin (100 μg/mL) and kanamycin (25 μg/mL). The overnight culture was diluted 1:10, grown to an A600 of 0.7, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and grown for an additional 4 hours at 37°C. Bacteria were pelleted by centrifugation (3000g, 10 minutes) and resuspended (5 g wet weight into 35 mL) in buffer 1 (0.1 M NaH2PO4, 10 mM 2-mercaptoethanol, and 10 mM Tris·HCl, pH 8.0) and DNase I (1 μg/mL). After 15 minutes at 4°C, the resuspended bacteria were disrupted twice using a French Press, and crude inclusion bodies were pelleted by centrifugation (10,000g, 15 minutes). Inclusion bodies were washed by resuspension in buffer II (1 M guanidine-HCl, 0.1% Tween-20, 10 mM 2-mercaptoethanol, and 10 mM Tris·HCl, pH 8.0) and repelleting. Washed inclusion bodies were dissolved at room temperature in buffer III (6 M guanidine-HCl, 0.1% Tween-20, 10 mM 2-mercaptoethanol, and 10 mM Tris·HCl, pH 8.0). Buffers I to III also contained a cocktail of protease inhibitors (antipain, 1 μg/mL, aprotinin, 1 μg/mL, pepstatin A, 1 μg/mL, phenylmethylsulfonyl fluoride, 1 mM; benzamidine, 1 mM). The supernatant was clarified twice by centrifugation (10,000g, 15 minutes) and then diluted 40-fold in buffer IV (500 mM NaCl, 10 mM 2-mercaptoethanol, 0.2% Tween-20, and 50 mM Tris·HCl, pH 8.0). The diluted lysate was loaded twice onto a PolyPrep column (Bio-Rad Laboratories, Richmond, CA) containing nickel-agarose (1 mL of 50% suspension washed with 25 mM Tris·HCl, 200 mM NaCl, pH 8.0) and allowed to elute by gravity. The column was washed extensively with buffer IV, and the fusion protein was eluted from the beads with 350 mM imidazole. The eluted proteins were dialyzed against buffer V (150 mM NaCl, 10 mM 2-mercaptoethanol, 0.05% Tween-20, and 25 mM Tris·HCl, pH 8.8), and the protein concentration was determined by the BCA assay (Pierce Biochemical, Rockford, IL).

Binding and internalization

Purified His-M195FANCF or His-FANCF was added to parental or CD33-transfected EUFA698 cells in RPMI 1640 with 10% FCS. After 10
minutes at 4°C, cells were washed and fresh medium was added in the absence of His-tagged proteins. Cells were then rapidly warmed to 37°C, and internalization of the fusion proteins was monitored by Western blotting and immunofluorescence microscopy, as described.14,16 Where indicated, chloroquine or brefeldin A (BFA) was added 30 minutes before the binding step and maintained throughout the experiment.

**Western blotting**

Cells (1 × 10⁶) were lysed directly in Laemmli buffer, subjected to electrophoresis on 10% polyacrylamide gels (SDS-PAGE), and transferred to PolyScreen membrane (NEN Life Science Products, Boston, MA). Blots were incubated with primary antibody in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20, and 5% nonfat dry milk, followed by incubation in the same buffer with peroxidase-conjugated goat anti-rabbit IgG (Gibco BRL, Grand Island, NY). Bands were detected by using enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). Densitometry was used to quantify the intensity of the bands on selected blots.

**Immunofluorescence microscopy**

HeLa cells were transiently transfected with pcDNA3-CD33. After 24 hours, cells were replated on glass coverslips and grown for an additional 24 hours before processing, as described.15 Nuclei were identified by Hoechst staining (Sigma, St Louis, MO).

**MMC sensitivity assay**

The response of FA lymphoblastoid cells to MMC was assessed by exposing parental or CD33-transfected cells (2 × 10⁵ cells/mL) to a range of concentrations of MMC in the presence or absence of His-M195FANCF (0.1 mg/mL). Viable cell numbers were determined after 6 days in culture by using trypan blue exclusion, as described.15 The concentration of MMC leading to 50% inhibition of cell viability (IC₅₀) was derived from the growth curves.

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**Results**

**Expression and purification of His-tagged FANCF**

We generated a single-chain antibody cDNA cassette based on the sequence of the variable portions of the anti-CD33 monoclonal antibody M195 (Figure 1A). The coding sequence of the resulting linker, N-Gly-Ser-Thr-Ser-Gly-Ser-Gly-Arg-Ala-Lys-Ser-Glu-Gly-Lys-Gly-C, and the relative orientation of the Vᵢ and Vᵧ chains were based on previous experience with other single-chain antibodies.23 The full-length FANCF cDNA was modified by insertion of a NotI site at the 5' end. The modified cDNA was then cloned downstream of M195 in the prokaryotic expression vector pQE9. Expression of His-M195FANCF in E. coli generated the expected 68.5-kd fusion protein, which was purified by single-step nickel-agarose chromatography (Figure 1B). The coding portion of full-length FANCF cDNA was also cloned downstream of the His tag to generate His-FANCF (data not shown). The final yield of purified His-M195FANCF was approximately 0.4 mg per liter of induced bacterial culture.

**CD33-mediated internalization of His-M195FANCF**

To evaluate the targeting function of the M195 single-chain antibody fused to FANCF, we stably transfected EUFA698 cells with CD33 before the protein transduction experiments. After 3 weeks in hygromycin B, surface expression of CD33 was ascertained by FACS (data not shown) and fluorescence microscopy (Figure 2). Parental and CD33⁺ cells were then incubated with His-M195FANCF, and the internalized fusion protein was detected by Western analysis (Figure 3). Internalization of His-M195FANCF was observed only in CD33⁺ EUFA698 cells, but not in parental CD33⁻ cells. Internalization was inhibited when cells were kept at 4°C, implicating an energy-dependent process. Internalization was also inhibited when cells were pretreated with trypsin to digest surface receptors. Finally, little if any internalization was observed with His-FANCF (data not shown), indicating that the M195 motif is essential for this function. These observations, taken together, strongly indicate that internalization of the fusion protein occurs by receptor-mediated endocytosis.

**Targeting of His-M195FANCF to the nucleus**

We next studied the binding and internalization of the fusion protein by fluorescence microscopy in HeLa cells. After a brief period of incubation with the fusion protein, membrane staining was observed for both CD33 and the fusion protein (Figure 2). After longer incubation times followed by permeabilization of the cell membrane with detergent, both proteins were found in association with vesicular structures resembling endosomes, but only the fusion protein was detected in the nucleus. FANCF has been shown to be localized primarily in nuclei.24 Therefore, these results demonstrate that the fusion protein is capable of entering the cell in association with CD33, after which it can be targeted appropriately to the nucleus.

**Role of Golgi in endosome-nuclear trafficking**

The ability of the fusion protein to be targeted to nuclei was used to evaluate the effect of different agents that can alter intracellular trafficking. For these experiments, we used a clone of HeLa cells stably expressing CD33 on the cell surface. Chloroquine can elevate the pH of acidic endosomes and inhibit uptake of extracellular proteins.23 Exposure of the CD33⁺ HeLa cells to 100 μM chloroquine significantly inhibited the appearance of the fusion protein in the nucleus (Figure 4). Cytoplasmic staining was also dimmer (data not shown) when compared with that in untreated controls. These observations further suggest that endosomes mediate the uptake of this fusion protein by CD33, but do not address the subsequent fate of the fusion protein. It is possible that the fusion...
protein enters the cytosol directly from endosomes. Alternatively, it may undergo retrograde transport and enter the cytosol through the Golgi and the endoplasmic reticulum. Both mechanisms have been described for exogenous proteins that are taken up by endosomes. BFA can disrupt the Golgi apparatus and inhibit retrograde transport. We exposed CD33+ HeLa cells to 1.6 μg/mL BFA and quantified the appearance of the fusion protein in nuclei. Nuclear staining was noted in approximately 80% of the cells, suggesting that BFA-sensitive retrograde transport is a minor route of transport of the fusion protein from endosomes into the cytosol and subsequently into the nucleus.

Intracellular turnover of His-M195FANCF

The intracellular half-life of His-M195FANCF was determined by incubation of CD33-transfected EUFA698 lymphoblasts with His-M195FANCF for 10 minutes at 4°C, followed by internalization and chase of the fusion protein for various time periods at 37°C. The cell lysates were analyzed by immunoblotting with anti-FANCF antibody. After the initial 10-minute pulse, the half-life of His-M195FANCF was estimated to be approximately 160 minutes (Figure 5). Although the half-life of endogenous FANCF has not yet been determined, this result demonstrates that the fusion protein is not rapidly degraded after uptake.

Functional complementation of FA group F cells

The effect of His-M195FANCF on cell survival was evaluated in FA lymphoblastoid cells from several different complementation groups. CD33+ and parental EUFA698 cells were exposed to a range of concentrations of MMC, and viable cells were quantified by trypan blue exclusion. CD33+ cells supplemented with His-M195FANCF at a concentration of 0.1 mg/mL, added at the beginning of the assay and supplemented daily, showed nuclear staining (data not shown) and were significantly more resistant to MMC than the parental cells (Figure 6A). This degree of resistance is comparable to that achieved by stable transfection of the EUFA698 cells with wild-type FANCF. To test the effect of the fusion protein on FA cells from other complementation groups, we also generated paired parental and CD33+ lymphoblastoid cells. Despite nuclear staining by the fusion protein (data not shown), no protection similar to that in CD33+ EUFA698 was noted in CD33+ FA groups C (HSC536) or A (HSC72) lymphoblastoid cells (Figure 6B). These observations demonstrate that CD33-mediated protein transfer can lead to functional correction of the major phenotypic defect in FA cells from the appropriate complementation group.

Discussion

As with gene transduction, many issues remain to be optimized with protein transfer, including the choice of ligand-receptor pairs, the efficiency of exit of the bioactive proteins from acidic endosomes, and the choice of target cells. The conclusions from our earlier proof-of-concept experiments involving the targeting of the IL-3 receptor with an IL-3–FANCC fusion protein can now be extended to include another ligand-receptor pair. In this case, the
The current study also provides additional mechanistic insights into the intracellular trafficking of such fusion proteins. Not surprisingly, chloroquine had a profound effect on the uptake and subsequent nuclear accumulation of the fusion protein. This effect is consistent with the known action of chloroquine: It inhibits the entry of exogenous proteins that use clathrin-dependent pathways for endocytosis by elevating the pH of acidic endosomes. BFA is a fungal drug that can disrupt the Golgi apparatus. Treatment of the CD33+ cells exposed to the fusion protein with BFA had a minor effect on the ultimate localization of the fusion protein to the nucleus. This observation suggests that the majority of the fusion protein is capable of entering the cytosol directly from endosomes, whereas a small fraction is subject to retrograde transport and enters the cytosol through the endoplasmic reticulum (Figure 7). It is possible that the single-chain M195 antibody is a surrogate ligand of CD33 rather than a conventional ligand. It should thus be possible to target CD33+ myeloid progenitor cells with this molecule fused to other bioactive proteins. Moreover, differences between the characteristics of the 2 FA proteins, FANCC and FANCF, provide additional grounds for optimism about the versatility of protein transduction. For example, the estimated molecular weight of FANCC is 63,424, whereas that for FANCF is 42,251. FANCF is also significantly more basic than FANCC (isoelectric points of 9.47 and 5.73, respectively), although the inclusion of the His tag and, to a lesser extent, the M195 motif could alter this parameter. Nevertheless, within the acidic milieu of the early endosome, the His-M195FANCF protein would be expected to have a net positive charge, which may be significantly different from the charged state of IL-3–FANCC. Both proteins are also hydrophobic, but the calculated hydrophobicity of FANCC is greater than that for FANCF. It is possible that the hydrophobic properties of these proteins facilitate their exit from endosomes. Also, our data show that FANCC is primarily cytoplasmic, whereas initial subcellular localization studies of FANCF suggest that it is primarily nuclear. The finding that the His-M195FANCF protein localizes to the nucleus after release from endosomes demonstrates that it is targeted correctly to the native subcellular compartment of FANCF. Finally, the intracellular half-lives of the 2 fusion proteins are also apparently different: The half life of the IL-3–FANCC protein is approximately 60 minutes, whereas that of the His-M195FANCF protein is approximately 160 minutes. Thus, despite several differences in the size, charge, localization, and half-life, receptor-mediated transfer was effective in both cases.

The current study also provides additional mechanistic insights into the intracellular trafficking of such fusion proteins. Not surprisingly, chloroquine had a profound effect on the uptake and subsequent nuclear accumulation of the fusion protein. This effect is consistent with the known action of chloroquine: It inhibits the entry of exogenous proteins that use clathrin-dependent pathways for endocytosis by elevating the pH of acidic endosomes. At least 2 pathways have been identified for the exit of exogenous proteins from acidic endosomes. BFA is a fungal drug that can disrupt the Golgi apparatus. Treatment of the CD33+ cells exposed to the fusion protein with BFA had a minor effect on the ultimate localization of the fusion protein to the nucleus. This observation suggests that the majority of the fusion protein is capable of entering the cytosol directly from endosomes, whereas a small fraction is subject to retrograde transport and enters the cytosol through the endoplasmic reticulum (Figure 7). It is possible that the
protein emerging from one or both transport routes is subsequently sorted to the nucleus to result in correction of the phenotypic defect in FA-F cells. Our experiments do not distinguish between these possibilities. Prolonged exposure of lymphoblasts to BFA is toxic and does not allow us to assess the sensitivity of these cells to MMC (unpublished observations, February 2001). Other strategies will be needed to further characterize the relative importance of these transport routes.

Although great strides are being made in the design of potent viral vectors and in overcoming various other biologic barriers to gene therapy, a number of problems remain to be solved before the broader implementation of gene therapy in clinical settings.1,2 FA is regarded as an excellent disease model for gene therapy, and many of these potential difficulties have been considered thoughtfully in this context.27 We have used fusion proteins to effect the transfer of bioactive proteins into FA cells through specific, endocytosis-competent receptors as an alternative or adjunct to gene transfer. Our data in these cell culture models need to be replicated in bone marrow cells from FA group F patients, but these are not currently available to us. If this strategy proves effective in appropriate preclinical models, it may be incorporated into future clinical trials with the aim of transient restoration of the function of FA cells.

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References

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