Clearance mechanism of a mannosylated antibody–enzyme fusion protein used in experimental cancer therapy

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MFECP1 is a mannosylated antibody–enzyme fusion protein used in antibody-directed enzyme prodrug therapy (ADEPT). The antibody selectively targets tumor cells and the targeted enzyme converts a prodrug into a toxic drug. MFECP1 is obtained from expression in the yeast Pichia pastoris and produced to clinical grade. The P. pastoris-derived mannosylation of the fusion protein aids rapid normal tissue clearance required for successful ADEPT. The work presented provides evidence that MFECP1 is cleared by the endocytic and phagocytic mannose receptor (MR), which is known to bind to mannose-terminating glycans. MR-transfected fibroblast cells internalize MFECP1 as revealed by flow cytometry and confocal microscopy. Immunofluorescence microscopy shows that in vivo clearance in mice occurs predominantly by MR on liver sinusoidal endothelial cells, although MR is also expressed on adjacent Kupffer cells. In the spleen, MFECP1 is taken up by MR-expressing macrophages residing in the red pulp and not by dendritic cells which are found in the marginal zone and white pulp. Clearance can be inhibited in vivo by the MR inhibitor mannan as shown by increased enzyme activities in blood. The work improves understanding of interactions of MFECP1 with normal tissue, shows that glycosylation can be exploited in the design of recombinant anticancer therapeutics and opens the ways for optimizing pharmacokinetics of mannosylated recombinant therapeutics.

Key words: Antibody-directed enzyme prodrug therapy/anti-carcinoembryonic antigen antibody MFE/clearance/ mannose receptor/Mannosylated antibody–enzyme fusion protein MFECP1

Introduction

Antibody-directed enzyme prodrug therapy (ADEPT) is a two-phase cancer treatment that uses a systemically administered antitumor antibody–enzyme conjugate to localize enzyme in tumors (Bagshawe 1987). After sufficient clearance from normal tissues, a prodrug is administered (Bagshawe et al. 1988; Bagshawe 1995) and is selectively converted into an active cytotoxic drug by enzyme at the tumor site. The first ADEPT system used in clinical trials employed a nonglycosylated chemical conjugate of the bacterial enzyme carboxypeptidase G2 (CP) from Pseudomonas sp. strain RS-16 and the F(αβ)2 fragment of A5B7, a monoclonal antibody to carcinoembryonic antigen (CEA) (Bagshawe 1995; Bagshawe et al. 1995; Napier et al. 2000). Persistence of this conjugate in blood necessitated the additional administration of a galactosylated antibody to CP in order to clear excess antibody–enzyme from circulation prior to administration of prodrug (Napier et al. 2000). Clearance of A5B7-CP was most likely via the galactose-specific hepatic asialoglycoprotein receptor (Sharma et al. 1994; Stockert 1995).

A subsequent and more direct approach for obtaining rapid clearance is to use a recombinant antibody–enzyme fusion protein produced in the methylotrophic yeast Pichia Pastoris, a eukaryotic microorganism which performs posttranslational glycosylation with mannose (Trimble et al. 1991; Breitmauer and Castellino 1999; Cereghino and Cregg 2000). A particular advantage of P. pastoris as opposed to Saccharomyces, which adds hypermannosylation to proteins, is the significantly shorter chain-length of the high-mannose structures. Furthermore, a number of P. pastoris products are already in clinical development (Herbst et al. 2002; Gergrgress 2004; Malkin et al. 2005; Kobayashi 2006) demonstrating feasibility and safety of using these in man.

We previously created a fusion protein of CP with MFE-23, a single-chain Fv antibody to CEA, and exploited P. pastoris mannosylation to clear the MFE-CP fusion protein from blood (Medzihradszky et al. 2004; Sharma et al. 2005) prior to administration of prodrug. The P. pastoris expressed nonclinical grade fusion protein of the CEA-binding antibody (MFE-CP) was shown to be glycosylated with high-mannose structures of 5–13 residues at two of the three potential N-glycosylation sites of each unit of the dimeric enzyme (Medzihradszky et al. 2004). Blood clearance of MFE-CP via liver was rapid leading to tumor-to-plasma ratios of over 1000:1 in human colon carcinoma xenograft models 6 h after administration (Medzihradszky et al. 2004; Sharma et al. 2005) and effective therapy in combination with a bisiodo-phenol mustard prodrug (Sharma et al. 2005). The first clinical grade MFE-CP, which is abbreviated to MFECP1, is made to GMP standards in P. pastoris and is currently in
Phase I/II clinical trials where similar hepatic clearance is observed (Chester et al. 2004; Mayer et al. 2004). We hypothesized that, in both mice and man, blood clearance of MFEC-CP is mediated by the mannose receptor (MR) interacting with P. pastoris-derived high-mannose structures.

MR is a member of the C-type lectin family (Taylor et al. 1990), and is an endocytic and phagocytic pattern recognition receptor that binds in a calcium-dependant manner to mannose, fucose, and N-acetylglucosamine terminating glycans (Largent et al. 1984). In liver, it is found on sinusoidal endothelium and Kupffer cells (Takahashi et al. 1998), where it acts as a molecular scavenger by clearing pathogenic microorganisms and endogenous glycoproteins with high mannose structures (Taylor et al. 2005). MR also plays an important role in cell activation and antigen presentation (Linehan 2005; Taylor et al. 2005; Martinez-Pomares et al. 2006) and, therefore, could be involved in the immune response to glycosylated protein therapeutics.

In the present study, we investigate the interaction of MFECPI with MR-expressing cells and provide in vitro and in vivo evidence that the antibody–enzyme fusion protein clears via MR. Furthermore, we show that the in vivo clearing cells are predominantly sinusoidal endothelial cells and not Kupffer cells. We also investigate uptake of MFECPI in potential antigen presenting cells (APC) in the spleen and show that this occurs by MR-expressing macrophages and not dendritic cells. Finally, we demonstrate that pharmacokinetics of MFECPI can be significantly modified by inhibition of MR in vivo.

Results

Analysis of MFECPI

MFECPI (clinical grade MFE-CP) was produced at a final yield of 80 mg. The purified material gave a characteristic profile for this protein when tested by size-exclusion chromatography (Figure 1A). Functional activity of the CP moiety was confirmed by testing for enzyme activity which was found to be 124 U/mg, where 1 U is the amount of enzyme required to hydrolyze 1 mmol of methotrexate per min at 37 °C.

N-glycan analysis of MFECPI

The degree of mannosylation of MFECPI was determined by DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis (DSA-FACE) analysis (Figure 1B). This analysis involves derivatisation of the PNGase F released carbohydrate chains with 8-amino-1,3,6-pyrenetrisulfonic acid and separation by polyacrylamide electrophoresis (Callevaert et al., 2001). Comparison of the profile of MFECPI with that of RNase B shows that Man8GlcNAc2 and Man9GlcNAc2 are the predominant carbohydrate structures (Figure 1B, Panels 2 and 3, respectively). In addition, higher mannosylated and lower mannosylated structures are also present, however, at a lower abundance than the Man9GlcNAc2 and Man10GlcNAc2 structures. This analysis agrees with previously published mass spectrometry data on MFE-CP (Medzihradszky et al. 2004).

The liquid chromatography mass spectrometry (LC/MS) analysis of trypsin digested glycopeptides identified two glycosylation sites, Asn-442 containing Man9GlcNAc2 to Man12GlcNAc2 structures and Asn-492 containing Man8GlcNAc2 to Man10GlcNAc2 structures.

Internalization in vitro of MFECPI by an MR-transfected fibroblast cell line

To determine whether MR has the ability to internalize the mannosylated MFECPI, an MR-transfected rat fibroblast cell was incubated with the antibody–enzyme fusion protein at 37 °C for various time intervals and analyzed by flow cytometry (Figure 2A, Table I). The cell line has previously been shown to mediate efficient endocytosis of mannose-BSA (Taylor et al. 1992; Taylor et al. 1990). Considerable fluorescence intensity and, hence, internalization was observed for MFECPI after 30 min when detected with an anti-CP antibody followed by R-phycoerythrin (R-PE) labeled IgG and compared to the omission of the anti-CP control experiment. The median fluorescence intensity shows that the highest internalization of MFECPI was observed after 45 min and internalization decreased at the 1-h and 5-h intervals. The fluorescence intensity represents only internal MFECPI because no fluorescence above background levels was observed when the cells were incubated with the antibodies prior to fixation (data not shown) to reveal surface binding. MFECPI is
expected to be removed from the cell surface during cell detachment with EDTA-trypsin due to the Ca\(^{2+}\) dependency of the carbohydrate–MR interaction. Addition of mannan at 37 °C to the fibroblast cell/MFECPI dish prevented internalization of the glycoprotein. Mannan is a known inhibitor of carbohydrate–MR interactions (Taylor et al. 1992) and, therefore, indicates that internalization of MFECPI is specifically mediated by MR.

When MFECPI was incubated at 4 °C for 1 h, the antibody-fusion protein was also not internalized, the observed fluorescence was comparable to the negative control. Furthermore, MFECPI was not internalized by rat-fibroblasts transfected with an empty vector (data not shown).

Confocal microscopy was used to visualize internal MFECPI (Figure 2B). MFECPI was incubated at 37 °C for 1 h with MR-transfected fibroblast cells described under Internalization in vitro of MFECPI by an MR-transfected fibroblast cell line. The cell was labeled with the cell tracer, carboxyfluorescein diacetate succinimidyl ester (CFSE). MFECPI was clearly visible inside the cell when revealed with Alexa Fluor® 594 labeled goat antimouse IgG. The nucleus was stained with Hoechst and the cell with CFSE. In (A) and (B), in the control experiments, the anti-CP was omitted.

### Table I. Quantification of internalization of MFECPI by MR-transfected fibroblast cells by flow cytometry

| Incubation time [min] | Measurement | Control | Δ Measurement – control |
|-----------------------|-------------|---------|-------------------------|
| 5                     | 5.77        | 5.69    | 0.08                    |
| 30                    | 10.56       | 6.22    | 4.34                    |
| 45                    | 18.55       | 5.60    | 12.95                   |
| 60                    | 18.29       | 5.63    | 12.66                   |
| 180                   | 14.52       | 4.70    | 9.82                    |
| 300                   | 11.77       | 4.02    | 7.75                    |
| 60 + Mannan           | 6.62        | 5.38    | 1.24                    |
| 60 at 4 °C            | 4.85        | 5.87    | −1.02                   |

*Fluorescence intensity (as geometrical mean) was measured after incubation of MFECPI for various time intervals with MR-transfected cells (at 37 °C if not otherwise indicated). Internalized MFECPI was detected with mouse anti-CP followed by R-phycocerythrin (R-PE) labeled goat antimouse IgG. For control experiments, the mouse anti-CP antibody was omitted. The intensities are representative of four independent experiments.

**Clearance and localization of MFECPI in vivo in liver**

Having established that MR can clear MFECPI in vitro in MR-transfected fibroblast cells, the role of MR in vivo clearance of MFECPI was studied in mice. MFECPI was injected i.v. in mice. The fluorescence images (Figure 3) of MFECPI-injected mice show localization of MFECPI in liver 5 min after intravenous injection of the fusion protein. Most of the fusion protein colocalizes with MR at this time point (Figure 3A), indicating that MFECPI is cleared also in vivo in liver by MR. Two cell types in the liver, sinusoidal endothelial cells and liver residence macrophages, Kupffer cells, express MR. In order to address whether there is a preference of clearance of MFECPI by one of these cell types, liver tissue was stained for CD31, an antigen highly expressed on endothelial cells. Merging of the images of CD31 and MFECPI staining showed considerable colocalization of these two molecules (Figure 3B and D). Therefore, MFECPI appears to be cleared by the endothelial cells. Staining of liver tissues with a pan macrophage antibody that recognizes the F4/80 antigen (expressed on almost all macrophages) shows distinct locations of Kupffer cells, and MFECPI and very little colocalization (Figure 3C). Hence, MFECPI appears to be cleared in liver predominantly by endothelial cells and not by the Kupffer cells although both cell types express MR. To substantiate this finding, the immunofluorescence staining was repeated and Kupffer cells were visualized using anti-CD68, an independent macrophage marker that recognizes the late endosomal glycoprotein macrosialin. Results obtained by merging of the image of CD68 staining with that of MFECPI showed that the majority of Kupffer cells do not colocalize with the enzyme-fusion protein (Figure 3E). In conclusion, endothelial cells are the preferred cell type involved in clearance of MFECPI in the liver.

**Localization of MFECPI in vivo in spleen**

Mechanisms of clearance of MFECPI were investigated in the spleen. Five minutes after i.v. injection of MFECPI, the
fusion protein accumulated in the spleen in the red-pulp area and uptake by MR and macrophages was investigated (Figure 4A). Merging of spleen images stained for MFECP1 and MR showed colocalization of the two proteins and that MR may be involved in clearance of MFECP1 in the spleen. Triple staining with the F4/80 recognizing pan macrophage antibody shows colocalization of MR, MFECP1, and macrophages, which accumulate in the red-pulp area of the spleen. Therefore, it appears that MR expressed on macrophages clears the antibody–enzyme fusion protein in the spleen. The spleen sections were also triple stained for MFECP1, MR, and dendritic cells in order to investigate whether these cells would be involved in clearing the fusion protein (Figure 4B). Dendritic cells are found in the marginal zone and in the white-pulp area of the spleen, in a different location to the MR-expressing macrophages. Dendritic cells in the spleen do not express MR and are not involved in clearing of MFECP1.

Inhibition of clearance of MFECP1 by mannan in vivo

Having established that MR is involved in the clearance of MFECP1 in the liver and spleen in vivo in mice, it was investigated whether prior addition of the known MR inhibitor mannan would inhibit clearance. Enzyme activity levels were measured in blood between 5 min and 24 h after MFECP1...
injection with or without prior intraperitoneal injection of mannan (Figure 5) using two cohorts of mice as described in the Materials and methods section. MFECP1 clearance in blood is very rapid and undetectable after 5 h without addition of mannan. At 5 min and 15 min after MFECP1 injection, the enzyme levels were comparable with or without addition of mannannan, mean values (of three mice) of 11.9 ± 1.0 with and 11.36 ± 0.7 without and 7.9 ± 0.5 without mannan, respectively. The later time points showed considerable increases in enzyme levels with prior addition of the MR inhibitor mannan, 2-fold after 30 min (7.1 ± 2.4 with and 3.5 ± 1.0 without mannan), 27-fold after 1 h (6.7 ± 0.3 with and 0.25 ± 0.02 without mannan), 75-fold after 3 h (4.64 ± 0.7 with and 0.062 ± 0.07 without mannan) and 206-fold after 5 h (4.1 ± 0.5 with and 0.02 without mannan). After 24 h, enzyme levels in blood fall below detection levels, also when mannan is added prior to MFECP1. Inhibition of blood clearance of MFECP1 by mannan provides further evidence of the specificity of clearance by MR and, in addition, shows that pharmokinetics of MFECP1 can be changed when MR is inhibited.

**Discussion**

This study investigates the clearance mechanism of a recombinant glycoprotein therapeutic used in experimental cancer therapy. The glycoprotein, MFECP1, is a fusion of a bacterial enzyme and an scFv antibody and would not be glycosylated in native form. However, expression in *P. pastoris* X33 strain has led to addition of high-mannose type oligosaccharides, which were found to be advantageous in achieving rapid clearance of the recombinant protein from noncancerous tissues in vivo. This is a prerequisite for prodrug treatment in ADEPT in which enzyme activity remaining in the blood can activate prodrug and cause systemic toxicity. It was hypothesized that clearance was mediated via MR in mice and man.

Our results demonstrate that MFECP1 is specifically internalized by human MR-transfected fibroblast cells in vitro and that internalization is blocked by the known MR inhibitor mannan. In vivo, we show by immunofluorescence that MFECP1 is specifically internalized by MR-expressing cells in the liver and spleen in mice. We also demonstrate that this uptake is blocked by pre-injection with mannan, since approximate 200-fold higher enzyme levels were measured in blood 5 h post administration of MFECP1 in the presence of the inhibitor compared to administration of MFECP1 alone.

Mannose is a preferred ligand for MR (Kery et al. 1992) and MFECP1 homo-dimer contains four N-linked high-mannose structures which were shown by tandem mass spectrometry to extend from 5 to 13 units in length in nonclinical grade MFE-CP (Herbst et al. 2002). In our current study, we demonstrate by DSA-FACE experiments that the same mannosylation pattern is present on the clinical product, MFECP1. MR uptake of proteins with similar mannosylation to MFECP1 has been reported by several other workers and in some cases has been shown to depend on monosaccharide density because efficient uptake was obtained by addition of at least 16 mannose residues. These studies have shown uptake of mannosylated albumins by MR in isolated perfused rat livers (Jansen et al. 1991), hepatic sinusoidal cells (Taylor et al. 1987) and in vivo in mice (Opanasopit et al. 2001).
Other glycoproteins, including RNase B have been shown to be rapidly cleared by alveolar macrophages, mediated by their terminating GlcNAc and/or mannose residues (Stahl et al. 1978). RNase B contains one N-linked glycosylation site of high-mannose type with 5–9 mannose residues (Joao and Dwek 1993).

In our studies, MFECP1 clearance occurred rapidly in liver in mice. MR is expressed in liver on two cell types, Fig. 4. MFECP1 colocalizes in the spleen with the mannose receptor (MR) and macrophages, which express MR (A) but not with dendritic cells, which do not express MR (B). Mice were injected i.v. with 0.3 mL MFECP1 (approximately 25 U) and tissue was removed after 5 min. The spleen sections were incubated with rabbit anti-CP followed by Alexa Fluor® 546 labeled goat antirabbit IgG, with Alexa Fluor® 488 labeled rat antimouse CD206 (MR) and with rat antimouse Pan macrophage antibody followed by goat antirat Alexa Fluor® 488 antibody (A). The spleen sections were also incubated with rabbit anti-CP followed by Alexa Fluor® 546 labeled goat antirabbit IgG, with Alexa Fluor® 488 labeled rat antimouse CD206 and with rat antimouse dendritic cell antibody and secondary Alexa Fluor® 647 labeled goat antirat antibody (B). Blue reveals nuclear staining by 4,6-diamidino-2-phenylindol. Images were revealed by fluorescence microscopy.
O-linked glycans colocalizes with MR in dendritic cells and O-linked region from crytococcal MP98, or both N- and (OVA) with either N-linked glycans, an attached S

outcome for MFECP1 in ADEPT, especially for multi-cycle treatment. Evasion of immune responses is a desired venting an immune response by re-directing clearance away from Kupffer cells. Mannosylating proteins might thus be a good strategy for pre-

sinusoidal endothelial cells and Kupffer cells as derived from the localization of internalized mannosylated ligands (Hubbard et al. 1979) and revealed by immunofluorescence with anti-MR and antimacrophage antibodies (Takahashi et al. 1998). However, our results showed that MFECP1 is predominately cleared by the endothelial cells and not the Kupffer cells. Although some reports have stated the opposite, our findings are consistent with most published work on clearance of mannosylated glycoproteins and neoglycoproteins. For example, in a pioneering study using iodinated mannosylated ligands, it was shown by electron microscope autoradiography that the radioactivity was predominantly associated with the endothelial cells and not the Kupffer cells (Hubbard et al. 1979). In an attempt to specifically target liver macrophages with mannose-terminal glucocerebrosidase in the lysosomal storage disorder, Gaucher’s disease, it was concluded that the major site of delivery were the endothelial cells and not, as attempted, the macrophages (Bijsterbosch et al. 1996). It might be concluded that addition of appropriate mannosylation is a good strategy if delivery to sinusoidal endothelial cells is a desired outcome. For example, the prevention of an immune response where the indigested foreign peptides are presented on major histocompatibility complex (MHC) molecules on Kupffer cells and activated T cells. Mannosylating proteins might thus be a good strategy for preventing an immune response by re-directing clearance away from Kupffer cells. Evasion of immune responses is a desired outcome for MFECP1 in ADEPT, especially for multi-cycle treatment.

A further site of MFECP1 uptake in vivo was the spleen. Here, two APC, macrophages, and dendritic cells, might be expected to be involved. Dendritic cells have been shown in vitro to use MR for internalization of mannosylated proteins and uptake by this route may be problematic for protein therapeutics as this can lead to increased immunogenicity. For example, P. pastoris-expressed mannosylated ovalbumin (OVA) with either N-linked glycans, an attached S/T-rich O-linked region from cystococcal MP98, or both N- and O-linked glycans colocalizes with MR in dendritic cells and can induce OVA-specific CD4\(^+\) T-cell proliferation (Lam et al. 2005). Furthermore, the approach has been successfully used to generate an antibody and T-cell response against MUC1 when the protein was linked to the MR ligand mannan and injected in patients with adenocarcinoma (Apostolopoulos and Mc Kenzie 2001). Tumor rejection, mediated by CD8\(^+\) T cells, was achieved with hydrophobized mannan nanoparticles that contained a fragment of the HER2 oncoprotein (Shiku et al. 2000).

Our in vivo results of spleen uptake showed that only macrophages express MR, and MFECP1 was only detected in macrophages. Providing more evidence, that uptake was MR mediated. The macrophage and dendritic cell distributions in the spleen were consistent with other reports where MR-expressing macrophages have been detected in the red pulp and not in the white pulp and marginal zone, whereas dendritic cells localize in the marginal zone and in the white pulp (Linehan et al. 1999). Because MFECP1 is not found in the dendritic cell residing marginal zone area, this might lead to a dampened immune response for this mannosylated antibody–enzyme fusion protein. This is consistent with its use in the clinic which showed no increase in immune response when compared to the nonmannosylated nonclinical grade MFE-CP (Mayer et al. 2004).

In conclusion and an advantage to ADEPT, the current study shows that MFECP1 is rapidly cleared in liver and spleen by MR and in both organs it is the cell type with the less immunological response, predominantly sinusoidal endothelial cells in liver and macrophages in spleen, that is involved in the clearance. To our knowledge, MFECP1 is the first recombinant therapeutic with N-linked high-mannose structures to be tested in man. Elucidating its mechanism of clearance is of relevance for clinical development of this and other mannosylated compounds. The finding that clearance can be inhibited by mannan leads the way to optimize pharmacokinetics of mannosylated recombinant therapeutics.

Materials and methods

Protein production and analysis

MFECP1 was produced in P. pastoris (strain X33) by fermentation interfaced with expanded bed adsorption immobilized metal affinity chromatography (EBA-IMAC) according to our standard protocols (Tolner et al. 2006a, 2006b). The 200-mM imidazole eluant from EBA-IMAC was concentrated 10-fold, dialyzed into PBS and purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare, Amersham, UK). The final product was applied to an endotoxin removal gel (Pierce Biotechnology, Rockford, IL), filter sterilized (0.2 μm, Nalgene), dispensed glass vials (GE Healthcare) and stored at −80 °C.

N-glycan analysis of MFECP1

N-glycan analysis of MFECP1 was performed by DSA-FACE analysis according to the procedure described previously (Callewaert et al. 2001).

Enzyme activity

The enzyme activity was expressed in units (U), where one unit is the amount of enzyme required to hydrolyze 1 mmol of MTX per minute at 37 °C. For concentrations of
2.5 U/mL or above, MFECP1 enzyme activity was quantified spectrophotometrically by measuring a change in absorbance at 320 nm due to CPG2 cleavage of methotrexate (MTX) (McCullough et al. 1971; Sharma et al. 2005). The rate of absorbance change was measured using a Hitachi U-2001 spectrophotometer with UV-Solutions software. Enzyme concentrations below 0.27 U/mL were measured using the sensitive high performance liquid chromatography (HPLC) assay using methotrexate as a substrate as described previously (Sharma et al. 2005).

**Cell culture**

Transfected rat fibroblast cell lines stably expressing the human MR and the empty vector (Taylor et al. 1990) (kindly provided by M.E. Taylor) were cultured in Dulbecco’s modified Eagle’s medium, containing L-glutamine (DMEM, Gibco, Invitrogen Cell Culture Products, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (BioWest, Nuillé, France), 10 U/mL penicillin and 10 mg/mL streptomycin (Cambrex, Nottingham, UK), and 100 μg/mL Geneticin (Gibco, Invitrogen Cell Culture Products) at 37 °C in a CO₂ humidified atmosphere.

**Flow cytometric analysis of internalization of MFECP1**

Aliquots of 1 × 10⁶ cells were preincubated at 4 °C in 35-mm² dishes with 10 μg MFECP1 in 500 μL DMEM medium containing 25 mM HEPES (pH 7.3) for 1 h with or without 0.5 mg of mannan (Sigma-Aldrich, Dorset, UK). Cells were transferred to a 37 °C humidified CO₂ atmosphere for 5, 30, 45 60, 180, or 300 min. After one wash with 1 mL PBS, cells were detached with 0.3 mL trypsin/EDTA (Cambrex) and added to 3 mL DMEM medium. Aliquots of 5 × 10⁵ cells were subsequently washed with 2 mL PBS/BSA and fixed with 100 μL fixation buffer (IntraStain fixation and permeabilisation kit, DakoCytomation, Dako, Glostrup, Denmark), washed with 2 mL PBS/BSA and treated with 100 μL permeabilization buffer in the presence of 1 μg mouse anti-CP, washed with 2 mL PBS/BSA and treated with 100 μL permeabilization buffer in the presence of 1 μg R-PE-conjugated goat antimouse IgG (BD Pharmingen, San Diego, CA) for flow cytometry analysis. In control experiments, the mouse anti-CP was omitted. Cells were maintained on ice prior to analysis by flow cytometry on an FACSCalibur™ cytometer with CellQuest software (Becton Dickenson, Cowley, UK) within no more than 12 h.

**Flow cytometric analysis of surface binding of MFECP1**

Cells were incubated with MFECP1 for 60 min and detached from the dish as described under Flow cytometric analysis of internalization of MFECP1. They were then incubated with 1 μg mouse anti-CP in 100 μL PBS at 4 °C for 60 min, washed with 2 mL PBS/BSA and treated with 1 μg R-PE-conjugated goat antimouse IgG in 100 μL PBS/BSA at 4 °C for 60 min and fixed with 100 μL fixation buffer. Analysis by flow cytometry is as described under Flow cytometric analysis of internalization of MFECP1.

**Immunofluorescence confocal microscopy of internalization of MFECP1**

Internalization of MFECP1 was also analyzed by confocal laser scanning microscopy. Cells were incubated with MFECP1 as described under Flow cytometric analysis of internalization of MFECP1 for 45 min at 37 °C in a humidified CO₂ atmosphere. This was followed by labeling of cells in PBS with 25 μM cell trace CFSE cell proliferation kit (Molecular Probes, Invitrogen) and incubation for 15 min at 37 °C, followed by incubation for 30 min at 37 °C in DMEM. Cells are detached with trypsin/EDTA as described under Flow cytometric analysis of internalization of MFECP1, fixed, permeabilized in the presence of 1 μg mouse anti-CP and then permeabilized in the presence of 1 μg Alexa fluor594 goat antimouse IgG (Molecular Probes, Invitrogen). Control experiments omitted the anti-CP antibody. The nuclei were stained with Hoechst 33258. Cytospins were prepared and mounted to glass slides with ProLong Gold anti-fade reagent (Molecular Probe, Invitrogen) and visualized using an Olympus® confocal scanning microscope (Olympus, London, UK).

**MFECP1 localization in mice**

Two hundred micrograms (equivalent to 25 U of CP), each of MFECP1 was injected “intravenously” via the tail vein in BALB/c mice. Five minutes post injection, the liver and spleen tissues were removed, frozen, subsequently stained, and examined by immunofluorescence microscopy.

**Immunofluorescence triple staining of MFECP1, MR, and dendritic cells in spleen.** Frozen cryostat sections were cut at 10 μm and fixed in acetone for 10 min. Sections were washed in PBS, blocked in 3% normal goat serum for 20 min. Staining was carried out using rabbit anti-CP antibody at 1/500, and rat antimonouse dendritic cell antibody (Hycult biotechnology, Uden, The Netherlands) at 20 μg/mL, both for 1 h at RT. MFECP1 was detected using an Alexa fluor® 546 goat antirabbit secondary antibody (Invitrogen) at 1/200 and dendritic cells with Alexa fluor® 647 goat antirat antibody (Invitrogen, 1/200), for 1 h at RT. Sections were stained for MR using rat antimonouse CD206 CD conjugated to Alexa fluor® 488 (Serotec, Oxford, UK) at 10 μg/mL for 1 h. Sections were counterstained for nuclei with 4′,6-diamidino-2-phenylindole (DAPI) mountant (Vector Laboratories, Burlingame, CA).

**Immunofluorescence triple staining of MFECP1, MR, and macrophages in the liver and spleen.** The methods described under Immunofluorescence triple staining of MFECP1, MR, and dendritic cells in spleen were used for MFECP1 and MR staining. Macrophages were stained with rat antimonouse pan macrophage antibody (recognizing F4/80 antigen) at 20 μg/mL (Hycult biotechnology) and secondary detection was with an Alexa fluor® 647 goat antirat antibody. Sections were counterstained for nuclei with DAPI as mentioned.

**Double staining of MFECP1, CD31, and MFECP1, macrophages in the liver.** The methods described under Immunofluorescence triple staining of MFECP1, MR, and macrophages in the liver and spleen were used for MFECP1 and macrophage staining. The liver sections, in addition, were
treated with rat antimouse CD31 at 1/2 (a gift from Professor A. Mantovani) followed by a goat antirat Alexa fluor® 488 secondary antibody. Sections were counterstained for nuclei with DAPI as under Immunofluorescence triple staining of MFECP1, MR, and dendritic cells in spleen.

**Triple staining of MFECP1, CD31 and CD68 in liver.**

The methods described under Double staining of MFECP1, CD31, and MFECP1, macrophages in the liver were used for staining of MFECP1 and endothelial cells with anti-CD31. The liver section was also stained with rat antimouse CD68 (a late endosomal macropage marker) conjugated to Alexa fluor® 647 (Invitrogen) diluted 10 μg/mL.

**MFECP1 blood clearance with and without addition of mann an in mice**

Six mice were injected i.v. into the tail vein with MFECP1 at a concentration of 200 μg in 0.3 mL (equivalent to 25 U of CP) per mouse. Six further mice were treated in the same manner but given 10 mg (0.3 mL, i.p.) each of mann an, 1 min prior to the addition of MFECP1. The mice were then divided into two cohorts of six mice. Each cohort contained three mice that had received MFECP1 alone and three mice which had received mann an prior to the injection of MFECP1. Cohort 1 was bled at 5 min, 30 min, 3 h, and 24 h, and cohort 2 was bled at 15 min, 1 h, 5 h, and 24 h. Twenty five microliters blood was collected at each instance. MFECP1 concentrations were measured as CP enzyme activity in plasma.

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**Conflict of interest statement**

None declared.

**Abbreviations**

ADEPT, antibody-directed enzyme prodrug therapy; APC, antigen presenting cells; CEA, carcinoembryonic antigen; CP, carboxypeptidase G2; DSA-FACE, DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis; EBA-IMAC, expanded bed adsorption immobilized metal affinity chromatography; LC/MS, liquid chromatography mass spectrometry; MFECP1, clinical grade fusion protein of the CEA-binding antibody, MFE and CP; MFE-CP, nonclinical grade fusion protein of the CEA-binding antibody, MFE and CP; MR, mannose receptor.

**References**

Apostolopoulos V, McKenzie IF. 2001. Role of the mannose receptor in the immune response. Curr Mol Med. 1:469–474.

Bagshawe KD. 1987. Antibody directed enzymes revive anti-cancer prodrugs concept. Br J Cancer. 56:531–532.

Bagshawe KD. 1995a. Antibody-directed enzyme prodrug therapy for cancer: its theoretical basis and application. Mol Med Today. 1:424–431.

Bagshawe KD. 1999b. Antibody-directed enzyme prodrug therapy: a review. Drug Dev Res. 34:220–230.

Bagshawe KD, Sharma SK, Springer CJ, Antoniw P. 1995. Antibody-directed enzyme prodrug therapy: a pilot scale clinical trial. Tumor Target. 1:17–29.

Bagshawe KD, Springer CJ, Searle F, Antoniw P, Sharma SK, Melton RG, Sherwood RF. 1988. A cytotoxic agent can be generated selectively at cancer sites. Br J Cancer. 58:700–703.

Bijsterbosch MK, Donker W, van de Bilt H, van Weely S, van Berkel TJ, Aerts JMFJ. 1996. Quantitative analysis of the targeting of mannose-terminal glucocerebrosidase. Predominant uptake by liver endothelial cells. Eur J Biochem. 237:344–349.

Brettbauer RK, Castellino FJ. 1999. Glycosylation of *Pichia pastoris*-derived proteins. Biotechnol Appl Biochem. 30 Pt 3:193–200.

Callewaert N, Geysen S, Molemans F, Contreras R. 2001. Ultra sensitive profiling and sequencing of *N*-linked oligosaccharides using standard DNA-sequencing equipment. Glycobiology. 11:275–281.

Cereghino JL, Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev. 24:45–66.

Chester K, Pedley B, Tolner B, Violet J, Mayer A, Sharma S, Boxer G, Green A, Nagl S, Begent R. 2004. Engineering antibodies for clinical applications in cancer. Tumour Biol. 25:91–98.

Gerngross TU. 2004. Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. Nat Biotechnol. 22:1409–1414.

Herbst RS, Hess KR, Tran HT, Tseng JE, Mullani NA, Charmsangavec J, Madden T, Davis DW, McConley DJ, O’Reilly MS, et al. 2002. Phase I study of recombinant human endostatin in patients with advanced solid tumors. J Clin Oncol. 20:3792–3803.

Hubbard AL, Wilson G, Ashwell G, Stukkenbrok H. 1979. An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of 125I-ligands among the liver cell types. J Cell Biol. 83:47–64.

Janssen RW, Molema G, Ching TL, Oosting R, Harms G, Moenlaar F, Hardonk MJ, Meijer DK. 1991. Hepatic endocytosis of various types of mannose-terminated carbohydrates. What is important, sugar recognition, net charge, or the combination of these features. J Biol Chem. 266:3343–3348.

Joao HC, Dwek RA. 1993. Effects of glycosylation on protein structure and dynamics in ribonucelease B and some of its individual glycoforms. Eur J Biochem. 218:239–244.

Kery V, Krepsinsky JJ, Warren CD, Capek P, Stahl PD. 1992. Ligand recognition by purified human mannose receptor. Arch Biochem Biophys. 298:49–55.

Kobayashi K. 2006. Summary of recombinant human serum albumin development. Biologicals. 34:55–59.

Lam JS, Mansour MK, Specht CA, Levitz SM. 2005. A model vaccine exploiting fungal mannosylation to increase antigen immunogenicity. J Immunol. 175:7496–7503.

Largent BL, Walton KM, Hoppe CA, Lee YC, Schnaar RL. 1984. Carbohydrate-specific adhesion of alveolar macrophages to mannosederivatized surfaces. J Biol Chem. 259:1764–1769.

Linehan SA. 2005. The mannose receptor is expressed by subsets of APC in non-lymphoid organs. BMC Immunol. 6:4.

Linehan SA, Martinez-Pomares L, Stahl PD, Gordon S. 1999. Mannose receptor and its putative ligands in normal murine lymphoid and nonlymphoid organs: in situ expression of mannose receptor by selected macrophages, endothelial cells, perivascular microglia, and mesangial cells, but not dendritic cells. J Exp Med. 189:1961–1972.

Malkin EM, Diemert DJ, McArthur JH, Perreault JR, Miles AP, Giersing BK, Mullen GE, Orcutt A, Muratova O, Awkal M, et al. 2005. Phase 1II trial of anti-CEA-derived Plasmodium falciparum malaria. Infect Immun. 73:3677–3685.

Martinez-Pomares L, Wiencek D, Stillion R, McKenzie EJ, Arnold JN, Harris J, McGreal E, Sim RB, Isacke CM, Gordon S. 2006. Carbohydrate-independent recognition of collagen by the macrophage mannose receptor. Eur J Immunol. 36:1074–1082.

Mayer A, Francis R, Sharma S, Sully C, Parker S, Tolner B, Griffin N, Germain M, Beckett P, Boxer G, et al. 2004. A phase I/II trial of antibody directed enzyme prodrug therapy (ADEPT) with MFECP1 and ZD2767P. Br J Cancer. 91 Supp 1:58.
McCullough JL, Chabner BA, Bertino JR. 1971. Purification and properties of carboxypeptidase G1. J Biol Chem. 246:7207–7213.

Medzihradszky KF, Spencer DI, Sharma SK, Bhatia J, Pedley RB, Read DA, Begent RH, Chester KA. 2004. Glycoforms obtained by expression in Pichia pastoris improve cancer targeting potential of a recombinant antibody–enzyme fusion protein. Glycobiology. 14:27–37.

Napier MP, Sharma SK, Springer CJ, Bagshawe KD, Green AJ, Martin J, Stumidity SM, Cushen N, O’Malley D, Begent RH. 2000. Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma. Clin Cancer Res. 6:765–772.

Opanasopit P, Shirashi K, Nishikawa M, Yamashita F, Takakura Y, Hashida M. 2001. In vivo recognition of mannosylated proteins by hepatic mannos receptors and mannan-binding protein. Am J Physiol Gastrointest Liver Physiol. 280:G879–G889.

Sharma SK, Bagshawe KD, Burke PJ, Boden JA, Rogers GT, Springer CJ, Melton RG, Sherwood RF. 1994. Galactosylated antibodies and antibody–enzyme conjugates in antibody-directed enzyme prodrug therapy. Cancer. 73:1114–1120.

Sharma SK, Pedley RB, Bhatia J, Boxer GM, El Emir E, Qureshi U, Tolner B, Lowe H, Michael NP, Minton N, et al. 2005. Sustained tumor regression of human colorectal cancer xenografts using a multifunctional mannosylated fusion protein in antibody-directed enzyme prodrug therapy. Clin Cancer Res. 11:814–825.

Shiku H, Wang L, Ikuta Y, Okugawa T, Schmitt M, Gu X, Akiyoshi K, Sunamoto J, Nakamura H. 2000. Development of a cancer vaccine: peptides, proteins, and DNA. Cancer Chemother Pharmacol. 46 Suppl: S77–S82.

Stahl PD, Rodman JS, Miller MJ, Schlesinger PH. 1978. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. Proc Natl Acad Sci USA. 75:1399–1403.

Stockert RJ. 1995. The asialoglycoprotein receptor: relationships between structure, function, and expression. Physiol Rev. 75:591–609.

Takahashi K, Donovan MJ, Rogers RA, Ezekowitz RA. 1998. Distribution of murine mannos receptor expression from early embryogenesis through to adulthood. Cell Tissue Res. 292:311–323.

Taylor ME, Bezouska K, Drickamer K. 1992. Contribution to ligand binding by multiple carbohydrate-recognition domains in the macrophage mannos receptor. J Biol Chem. 267:1719–1726.

Taylor PR, Gordon S, Martinez-Pomares L. 2005. The mannos receptor: linking homeostasis and immunity through sugar recognition. Trends Immunol. 26:104–110.

Tolner B, Smith L, Begent RHJ, Chester KA. 2006a. Expanded bed adsorption immobilized affinity chromatography. Nat Protoc. 1:1213–1222.

Tolner B, Smith L, Begent RHJ, Chester KA. 2006b. Production of recombinant protein in Pichia pastoris by fermentation. Nat Protoc. 1:1006–1021.

Trimble RB, Atkinson PH, Tschopp JF, Townsend RR, Maley F. 1991. Structure of oligosaccharides on Saccharomyces SUC2 invertase secreted by the methylotrophic yeast Pichia pastoris. J Biol Chem. 266:22807–22817.