Molecular and functional characterisation of a fusion protein suited for tumour specific prodrug activation

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Summary A fusion protein consisting of the humanised Fab fragment of the anti CEA MAb BW 431 and the human β-glucuronidase was expressed in BHK cells. Functional testing revealed that the specificity and avidity of the humanised V region was similar to the original murine MAb BW 431. Furthermore, the enzymatic activity, pH sensitivity and stability of the human β-glucuronidase in the fusion protein was comparable to the activity of recombinant human β-glucuronidase. Using anti-idiotype affinity chromatography, two molecules of a molecular weight of 125 kDa or 250 kDa could be visualised under nonreducing conditions in SDS-PAGE. Reducing conditions revealed a 25 kDa light and 100 kDa heavy chain. Due to its suitable biological characteristics this fusion protein might be an appropriate molecule allowing a site specific antibody directed enzyme prodrug therapy (ADEPT) in vivo.

Currently, the treatment of non-disseminated solid tumours is performed by surgery and irradiation. Both methods can be considered to be relatively tumour selective, without significantly harming the rest of the body. If however the tumour has disseminated to various organ sites, the metastases can be treated by chemo- or hormone therapy only. Chemotherapy has considerable side effects and a minor influence on patients’ survival due to the lack of specificity of action or the induction of resistance. Hormone therapy alone has a limited tumour spectrum.

To overcome these obvious limitations of today’s treatment modalities, we tailored a fusion gene consisting of the Vh and Cα1 Exons of a humanised MAb and the human β-glucuronidase cDNA (Lorenz et al., 1991). The product encoded by the fusion gene might be suitable for performing an antibody directed enzyme prodrug therapy (ADEPT). The concept of ADEPT as developed by Philpott et al. (1973a,b; 1974) and reemphasised by Bagshawe (1987) and Bagshawe et al. (1988) assumes that an antibody enzyme conjugate after selective localisation at the tumour target site, and its clearance from normal tissues, activates a nontoxic low molecular weight prodrug to a highly toxic drug in the tumour by enzymatic catalysis.

The therapeutic success of this approach depends on several factors:

(a) The stability of the prodrug in vivo.
(b) The difference in toxicity between prodrug and drug.
(c) The molar toxicity of the drug.
(d) The pharmacokinetics of the prodrug.
(e) The tumour selectivity of the antibody enzyme conjugate.
(f) The turnover rate of the enzyme used in the conjugate.
(g) The molecular weight and pharmacokinetic of the antibody enzyme conjugate.
(h) The percolation and retention of the antibody enzyme conjugate at the tumour site and its elimination kinetics from normal tissues.
(i) The concentration of free unconjugated endogenous enzyme.
(j) The immunogenicity of the antibody enzyme conjugate.

Despite the obvious complexity of the system, a first clinical trial with a Fab(’), fragment of a murine anti CEA MAb chemically linked to carboxypeptidase G2 (CPG2) from Pseudomonas origin combined with a para-N (mono-2-chloro-ethyl monomesy) amino benzoyl glutamic acid prodrug was performed (Bagshawe, 1991). Five patients with advanced colorectal carcinomas were treated. In two out of five patients, regression of CEA expressing metastases was observed in addition to minor responses in three out of five patients. Treatment became inefficient due to the fast activation of a neutralising anti CPG2 immune response. This trial shows that the ADEPT concept works in the patient, but has to be optimised to obtain better therapeutic effects.

Our approach to the ADEPT concept is based on the availability of the high avidity anti CEA MAB BW 431 (Bosslet et al., 1988), its CDR-grafted (Jones et al., 1986; Riechmann et al., 1988) humanised version (Güssow & Seemann, 1991) and the cloned cDNA forhuman placental β-glucuronidase (Oshima et al., 1987), a lysosomal endogenous enzyme (Brot et al., 1978). Out of these human building blocks a fusion gene was constructed (Lorenz et al., 1991). This construction was performed in analogy to the original work of Neuberger’s group (Neuberger et al., 1984; Williams & Neuberger, 1986) who showed that a fusion protein between a mouse Fab and nuclease or polymerase could be functionally expressed. In the present paper the molecular and functional characteristics of a fully humanised/human fusion protein expressed in BHK cells will be reported.

Materials and methods

Recombinant DNA-techniques

Construction and cloning of the fusion gene was done by standard recombinant DNA techniques (Sambrook et al., 1989) as described by Lorenz et al. (1991b).

DNA-transfection

BHK cells were transfected with plasmid DNA by Ca-phosphate coprecipitation (Graham & van der Eb, 1973). Supernatants of BHK cells growing in selection medium were tested for functionally active fusion protein using the fusion protein activity assay described below.

Fusion protein activity assay

Polystyrol U-bottom microtiter plates were coated with 0.1–0.5 µg CEA or mucin. After blocking with 1% casein in PBS, pH 7.2, 50 µl of BHK transfecoma supernatant were added and removed after 30' of incubation at RT. After extensive washing of the plates, 50 µl of a 2.5 mM 4-methylumbelliferyl-β-D-glucuronide solution in 200 mM sodium acetate buffer,
pH 5, was added and incubated for 2 h at 37°C. The reaction was stopped using 0.2 M glycine + 0.2% SDS, pH 11.1. Fluorogenic units (FU) were determined as described by Glaser and Sly (1973).

**Avidity determination**

Avidity of fusion protein to purified CEA was determined according to Tipton et al. (1990). The avidity to cell bound CEA was measured as described by Bosslet et al. (1988).

**Histochemical and immunohistochemical tissue specificity**

Transfectoma supernatants containing fusion protein were added to cryopreserved tissue sections from various human tissues for 30' at RT. After washing the selectively bound fusion protein was histochemically detected via its enzymatic catalysis resulting in a red insoluble stain as described by Murray et al. (1989) or visualised immunohistochemically using an alkaline phosphatase labelled goat anti β-glucuronidase antibody.

**Purification and analysis of fusion protein**

BHК transfected supernatants containing the fusion protein were purified by anti-idiotype affinity chromatography (Bosslet et al., 1991). Adsorption was performed at pH 7.2, elution was done at pH 4. Samples of the elution peak were analysed by SDS-PAGE under reducing or nonreducing conditions followed by silverstaining (Poehling & Neuhoff, 1981) or Western blotting (Towbin & Gordon, 1979).

**Results**

**Short description of the DNA constructs**

A fusion gene was constructed consisting of the human IgG promoter region and signal peptide exon, the humanised version of the rearranged V₃ gene of MAB BW 431, the C₂₁ exon of human IgG₃, one hinge exon derived from human IgG₁, a synthetic linker peptide and the cDNA coding for the mature human β-glucuronidase. This construct was inserted via its HindIII and Sall restriction sites in the pAB-vector (Chart 1) (Zettlmeissl et al., 1987).

A second pAB-vector containing the gene for the humanised light chain of MAB BW 431 is depicted in chart 2. The light chain gene contains the humanised V₃-gene of the L-chain with IgG₃ promoter region and signal exon and the human C-kappa gene. These two vectors were cotransfected into BHК cells with the plasmid pRMH 140, carrying a neomycin resistance gene (Hudziak et al., 1982) and the plasmid pSVdhfr (Subramani et al., 1981) carrying the dihydrofolate reductase gene allowing positive selection with methotrexate (Wirth et al., 1988).

**Detection of fusion protein secreting transfectomas**

BHК cells were transfected by Ca-phosphate co-precipitation (Graham & van der Eb, 1973) using the four independent plasmids outlined above. Supernatants from transfectoma clones grown in selection medium containing G418 and methotrexate were evaluated in the fusion protein activity assay (see Materials and methods). Out of 97 transfectomas investigated eight transfectomas secreted detectable amounts of molecules with CEA binding potential and β-glucuronidase activity.

**Purification and analysis of fusion protein**

Supernatants from BHК transfectedoma cells were processed through an anti-idiotype affinity column as described in Materials and methods. Samples collected from the elution peak at pH 4 were analysed by SDS-PAGE under reducing and nonreducing conditions followed by silverstaining. The SDS-PAGES presented in Figure 1 reveal two bands with an apparent MW of ~100 kDa and ~25 kDa under reducing conditions and two major bands of a MW of ~250 kDa and ~125 kDa under nonreducing conditions. A minor band of a MW of 200 kDa was detected as well.

Western blotting of reduced SDS-PAGE using a horse radish peroxidase labelled (HRP) goat anti human κ antisem as a detection system revealed a 25 kDa band. Under nonreducing conditions both a 125 kDa and a 250 kDa band were visualised. A 100 kDa band was detected under reducing conditions if a HRP labelled goat anti human β-glucuronidase antisem was used for immunostaining. Nonreducing conditions revealed two major bands at 125 and 250 kDa after staining with the HRP labelled goat anti human β-glucuronidase antisem. Furthermore, TSK 3000 gel chromatography of native eluate revealed a single major peak of β-glucuronidase activity at a molecular weight position of approximately 250 kDa (data not shown).

**Avidity determination of the purified fusion protein**

The strength of binding of the purified native fusion protein to purified CEA as well as to cell-associated CEA was determined in comparison to MAB BW 431 or its humanised version. The association constants as determined for the three molecules by two independent methods were in the range of 10⁹ l mol⁻¹ and did not show significant differences (Table I).
Histochemical and immunohistochemical evaluation of the tissue specificity

The avidity evaluation proved that the binding capacity of the fusion protein to purified CEA or CEA bearing cell lines was unchanged compared to the original MAb BW 431 or its humanised version. To further investigate the properties of the fusion protein’s binding region we evaluated its histochemical and immunohistochemical specificity on cryopreserved human tissue sections. The data collected from several independent experiments are presented in Table II. Histochemical investigations using naphthyl β-glucuronide combined with hexazotised pararosaniline as substrate for the β-glucuronidase (Murray et al., 1989) revealed that the fusion protein binds to all colon and stomach carcinomas and is enzymatically active. Normal lung and brain are not stained, as expected. Normal liver and kidney are weakly stained due to endogenous lysosomal β-glucuronidase also detectable in the MAb BW 431 negative control (lanes 1 and 2). Furthermore the immunohistochemical specificity using an AP-labelled goat anti human β-glucuronidase antibody or an AP-labelled goat anti mouse Ig antibody showed an identical staining reaction. The colon and stomach carcinomas were positive, whereas the normal tissues like lung, liver, kidney and brain were negative as reported for MAB BW 431. Again a weak reaction due to binding of the anti β-glucuronidase antibody to endogenous β-glucuronidase was observed (lanes 3 and 4). These data clearly demonstrate that the fusion protein has a specificity similar or identical to that of the original MAB BW 431.

Table I Comparison of the avidity of the murine MAb BW 431 with its CDR-grafted humanised version and the fusion protein

|           | $k_a \times 10^{6}$ (Mol)$^{-1}$ |
|-----------|----------------------------------|
| mu BW 431 | 3.0 - 4.7$^a$                    |
| hu BW 431 | 1.0 - 2.2$^a$                    |
| hu 431 β-gluc | 1.3 - 2.0$^a$                 |

$^a$Tipton et al. (1990); $^b$Boslet et al. (1988).

Influence of pH on the catalytic rate of the fusion protein

After the purification of the fusion protein and the determination of its binding potential to purified CEA the turnover rate at various pH values was determined. Figure 2 shows the pH dependence of the fusion protein bound to solid phase attached CEA as revealed by the fusion protein activity assay.

The maximal catalytic rate was observed at pH 4.5. At pH 7.4, the pH in normal tissues, the catalytic rate is about 10% of the optimal value. At pH 7.0, a pH reported for solid tumours (Tannock & Ratlin, 1989) the catalytic rate is about 20% of the maximal activity. After adjustment of pH from 7.4 to 4.5 the fusion protein revealed its full catalytic potential arguing for its stability (data not shown).

Determination of the turnover rate of the fusion protein

With the help of the fusion protein activity assay, a linear correlation between incubation time, fusion protein concentration and liberated fluorogenic units (FU) could be demonstrated (Figure 3). One thousand FU corresponded to 0.14 µg methylumbelliferone (MU), the liberated fluorogenic label. Based on this correlation the turnover rate of the fusion protein was determined. At pH 7.0, 1 Mol of CEA bound

![Figure 1](image1.png)

Figure 1 Photograph showing two silverstained SDS-PAGES. Lanes A represents molecular weight markers. Lane B shows the purified fusion protein under reducing conditions, Lane C under nonreducing conditions.

![Figure 2](image2.png)

Figure 2 Fluorogenic units (FU) are plotted against the pH value. Values obtained with 500 ng ——, 83 ng —— or 16 ng —— of fusion protein are presented and show a similar type of curve. konz. ——; 1/6 ——, 1/32 ——. Incubation 2 h of 37°C.

Table II Histochemical and immunohistochemical evaluation of the tissue specificity of the fusion protein compared to MAB BW 431

| Tissues investigated | Histochemical substrate reaction using naphthyl β-glucuronide and hexazotised pararosaniline | Immunohistochemical substrate reaction using AP-labelled |
|----------------------|-------------------------------------------------------------------------------------------|-------------------------------------------------------|
|                      | fusion protein positive/tested | MAB BW 431 positive/tested | fusion protein positive/tested | MAB BW 431 positive/tested |
| Carcinomas           |                                |                          |                          |                          |
| Colon Car            | 7/7                           | 0/7                      | 7/7                      | 7/7                      |
| Stomach Car          | 4/4                           | 0/4                      | 4/4                      | 4/4                      |
| Normal tissues       |                                |                          |                          |                          |
| Lung                 | 0/3                           | 0/3                      | 0/3                      | 0/3                      |
| Liver                | 4/3                           | 4/3                      | 4/3                      | 4/3                      |
| Kidney               | 2/2                           | 2/2                      | 2/2                      | 2/2                      |
| Brain                | 0/2                           | 0/2                      | 0/2                      | 0/2                      |

$^a$Endogenous intralysosomal β-glucuronidase detectable in sections without fusion protein also.
fusion protein cleaves $\approx 12,000$ moles of methylumbelliferyl $\beta$-glucuronide, the synthetic prodrug in 1 h at 37°C. Similar turnover rates were observed using a prodrug based on daunomycin linked via a urethane nitrophenyl spacer to $\beta$-glucuronic acid (manuscript in preparation).

**Discussion**

This study demonstrated that the molecular construct consisting of the binding region of the humanised anti CEA MAb 431 and the human endogenous lysosomal enzyme $\beta$-glucuronidase could be expressed and purified to homogeneity as a highly effective fusion protein. Using the 'fusion protein activity assay' we were able to detect eight BHK transfectomas secreting significant amounts of fusion protein that bind to CEA and catalyse the cleavage of the 4-methylumbelliferyl $\beta$-glucuronide prodrug to 4-methylumbelliferone and glucuronic acid due to the fusion protein's $\beta$-glucuronidase activity. From transfectoma supernatants two molecules could be isolated using anti-idiotype affinity chromatography. Under denaturing conditions, one molecule represented a monovalent protein and the other consisted of a bivalent protein as revealed by our SDS-PAGES and Western blotting data (Figure 1). A schematic diagram of the two molecules is given in Figure 4a. The monovalent molecule (Figure 4b) of a MW of 125 kDa contains the light chain of the humanised MAb BW 431 consisting of the $V_L$ and $C_L$ domains covalently linked by an interchain disulphide bond to the humanised heavy chain. The humanised heavy chain is built up by the $V_H$ and $C_H1$ domain of the humanised MAb BW 431 the N-terminal part of the human IgG1 hinge region, a linker peptide and the human $\beta$-glucuronidase. In this monovalent molecule the two hinge region cysteins form an intrachain disulphide bond. If the hinge region cysteins form two interchain disulphide bonds a bivalent fusion protein arises (Figure 4c) which can be isolated from transfectoma supernatants at a similar amount as the monovalent fusion protein (Figure 4c). Under native conditions the two molecular forms exist as bivalent molecules (Figure 4d) as shown by gel chromatography under non denaturing conditions.

Furthermore, the mild elution conditions applicable to the anti-idiotype affinity chromatography resulted in the isolation of a protein fraction with satisfying biochemical properties.

**Figure 3** Fluorogenic units (FU) are plotted against the incubation time at 12.5 ng $\cdots$, 2.5 ng $\cdots$ or 0.25 ng $\cdots$ of fusion protein. A linear time-activity relationship was found at all three fusion protein concentrations.

**Figure 4** A schematic diagram of the molecular species of fusion protein secreted from transfectoma supernatant is shown. a, Fusion protein molecules found under denaturing conditions after isolation using anti-idiotype affinity chromatography. b, Diagram of the fusion protein monomer showing the building blocks of the construct. c, Diagram of the fusion protein dimer with covalent linkage by two interheavy chain disulphide bonds. d, Diagram of two fusion protein dimers with and without interheavy chain disulphide bonds. Both molecules are found under native conditions after isolation using anti-idiotype affinity chromatography.
Avidity to CEA was found by two independent methods to be in the range of 10^9 Mol⁻¹, a figure similar to the avidity of the murine MAB BW 431 or its humanised version (Table I). Since the molecular forms of the fusion protein as revealed under denaturing conditions are associated in the native state forming molecules with two identical binding sites, the avidity measurements refer to the binding strength of bivalent molecules. In addition, the histochemical and immunohistochemical tissue specificity of the fusion protein was not distinguishable from that of the murine MAB BW 431 (Table II). Not only the binding portion of the fusion protein was functionally active, but also the enzyme moiety showed a catalytic activity and pH profile similar to human β-glucuronidase (Brot et al., 1978). Despite the fact that the catalytic optimum of the fusion protein is at pH 4.5 and the turnover rate at pH 7.0, a pH value assumed for human solid tumours (Tannock & Ratin, 1989), is about 20% of the optimal rate it still seems to be high enough to fulfill the assumptions made in the ADEPT concept. Due to its fully human or humanised building blocks, the fusion protein should have a low immunogenicity in humans, if at all. This characteristic should allow the long term application of this molecule in humans and distinguishes this construct from xenogeneic mouse antibody - bacterial enzyme conjugates which were reported to elicit a fast neutralising antibody response in the patient (Bagshawe et al., 1991). Before the fusion protein can be considered for clinical evaluation, its pharmacodynamic (Natowicz et al., 1979) in relation to the carbohydrate content (Stahl et al., 1976) and therapeutic effects combined with appropriately designed prodrugs (Stella et al., 1985) must be studied in further preclinical model systems. Prodrug systems which are investigated presently are glucuronides of daunomycin and adriamycin. To our knowledge, this is the first report, describing a functionally active fusion protein, consisting of a humanised tumour selective binding portion and a human lysosomal enzyme with potential applicability for a more selective tumour therapy.

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