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An antigenic HIV-1 peptide sequence engineered into the surface structure of transferrin does not elicit an antibody response

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Abstract One novel approach for the biological delivery of peptide drugs is to incorporate the sequence of the peptide into the structure of a natural transport protein such as human serum transferrin (HST). However, a potential drawback is that the HST may increase the immunoreactivity of the peptide, in the same way that carrier proteins can be used to generate highly immunogenic peptide hapten conjugates. In this study we have generated a recombinant HST carrier protein that contains a peptide substrate of HIV-1 protease (VSQNYPIVL). The protein retained native HST function, and the peptide was surface exposed since it was immunoreactive in native dot blots, and was cleaved by HIV-1 protease. Immunisation of rabbits with the recombinant protein elicited only a very poor anti-peptide immune response. In contrast, strong anti-peptide immune responses were raised against both the peptide alone, and a chemical conjugate of the peptide with HST. These data demonstrate that it is possible to attenuate the immune response normally directed against an immunogenic peptide sequence by engineering into a surface exposed loop of HST. These findings may have an important impact on the future design of peptide delivery systems.

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Key words: Transferrin; Peptide delivery; Antibody

1. Introduction

Human serum transferrin (HST) is a monomeric glycoprotein with a molecular mass of around 80 kDa, and is able to bind tightly, but reversibly, two ferric irons together with two bicarbonate co-ions. It has two roles. First, it mediates the transport and uptake of iron into cells. Second, it regulates the availability of free iron in the body fluids, preventing the production of potentially toxic free radicals and providing bacteriostatic functions.

In recent years, several studies have demonstrated that the transferrin uptake pathway is useful for delivering drugs into transformed and activated cells. These cells express high levels of transferrin receptors because they have a high requirement for iron. Particularly, Laske and co-workers showed that HST/diphtheria toxin conjugates are effective at eradicating human glioma tumors in mice [1]. Treatment produced on average of 95% regression in tumour volume after 30 days. In contrast, tumour volumes in animals treated with free toxin increased by about 300%, and by 1000% in the controls. A clinical trial has demonstrated that this conjugate is also effective in humans [2]. Patients with recurrent malignant brain tumours received the conjugate intra-cerebrally using high-flow interstitial microinfusion. Two of the 15 treated patients showed complete remission, and nine showed a 50% reduction in tumour volume, as determined by nuclear magnetic resonance imaging. In none of the patients were there any signs of systemic toxicity. These results clearly demonstrate that this is an effective approach for drug delivery.

Recently, we proposed an alternative, novel way to use the transferrin uptake pathway for the cellular delivery of therapeutic peptide drugs [3]. Instead of using chemical conjugation to join the peptide drug to transferrin, we reasoned that it should be possible to incorporate the drug into the structure of transferrin using recombinant protein engineering. The short biological half-life of peptide drugs is often a major problem and those which are water-soluble usually do not penetrate the cell wall readily. A recombinant transferrin analogue, containing the therapeutic peptide within the structure would bypass these problems.

Surface exposed loops of globular proteins can frequently tolerate insertions of additional amino acids without altering the function of the protein [4]. Therefore, it should be possible to introduce peptide sequences into the surface of transferrin without destroying function. As such, the peptides would 'hide' in the surface of the transferrin analogue, be actively uptaken into the cell, and then recognised (i.e. be functional) at the site of inhibitory activity. In this scenario, the function of the transferrin molecule would be analogous to that of a 'Trojan Horse' for the delivery of the peptide sequence into the cell. To demonstrate proof of principle, we generated functional transferrin analogues targeted to the protease of HIV-1; the etiological agent of AIDS. In this study, a peptide substrate (VSQNYPIVL) of HIV-1 protease was introduced into a surface exposed loop of HST. The HST analogue retained native transferrin function and could be cleaved by HIV-1 protease [3].

Although our study demonstrated that HST could be used for the biological delivery of a peptide [3], we did recognise one potential drawback; it is possible that the HST could enhance the immunogenicity of the peptide, in the same way that carrier proteins can be used to generate highly immunogenic peptide hapten conjugates. In the present study we chose to examine the immunogenicity of the HST analogue. Immunisation of rabbits with the recombinant protein elicited only a very poor anti-peptide immune response. In contrast, strong

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anti-peptide immune responses were raised against both the peptide alone, and a chemical conjugate of the peptide with HST. These data demonstrate that it is possible to attenuate the immune response normally directed against an immunogenic peptide sequence by engineering into a surface exposed loop of HST. These findings may have an important impact on the future design of peptide delivery systems.

2. Materials and methods

2.1. Reagents

Native HST was obtained from Sigma (Vienna, Austria) and Boehringer Mannheim (Vienna, Austria). Peptides were provided by Genosys (Cambridge, UK), who also synthesised the HST/HIV-1 protease cleavage site peptide conjugate. Antisera were produced in rabbits using standard techniques.

2.2. Recombinant proteins

Production of the recombinant proteins used in this study has been described in an earlier paper [3]. Essentially, molecular modelling was used to identify an insertion site (amino acid 289 in the transferrin sequence) which was located in a surface exposed loop of the protein, and which was distant from biologically active domains. A nucleotide insertion coding for the HIV-1 protease cleavage site VSQNYPIVL was then cloned into this site using recombinant PCR. This generated a mutant, termed HST M289. These proteins were then expressed to high levels using a baculovirus expression vector system, and purified from cell supernatants to homogeneity using column chromatography. We demonstrated (i) that the proteins retained native HST function, (ii) that the peptide cleavage site engineered into M289 was surface located as predicted, and (iii) that inserted peptide sequence in M289 was cleaved by HIV-1 protease.

2.3. Analysis of immunogenicity of protease cleavage site in M289

Rabbits were immunised using standard procedures three times at monthly intervals with the M289 analogue, a conjugate of HST and the HIV-1 protease cleavage site peptide, or the peptide alone. After 4 months, rabbit serum was collected, and titres of antibodies specific for the HIV-1 protease cleavage site or HST assayed using an antibody capture ELISA.

An antibody capture ELISA was established according to standard methods. The wells of an ELISA plate (Maxisorb, Nunc) were coated with the peptide (20 ng/well) or HST (40 ng/well) for 3 h at room temperature, non-specific binding sites on the plate blocked with either 0.1% (v/v) Tween-20 in PBS (PBS-T) or 3% (w/v) bovine serum albumin in PBS overnight at 4°C. After extensive washing in PBS-T, serum samples (100 μl) were applied to the ELISA plate wells, and incubated for 1 h at 37°C to allow antigen capture. For detection, wells were treated first with a biotinylated primary detection antibody (100 μl biotinylated rabbit anti-HST polyclonal, diluted 1:5000 in PBS-T) and then with a streptavidin-horse radish peroxidase (HRP) conjugate (Amersham; 100 μl of 1:50 000 dilution in PBS-T). A colourimetric HRP substrate mix (Turbo TMB, Pierce Biochemicals; 100 μl) was then added to the ELISA plate wells and the colour allowed to develop for 35 min before the addition of the stop reagent (4 M sulphuric acid). ELISA plates were then read at 695 nm.

3. Results

3.1. Immunogenicity of HST analogues

If HST analogues are to be useful as drugs, then the foreign peptide contained within the molecule should be neither immunogenic, nor alter the antigenicity of the HST carrier molecule. The ideal experiment to test this would be to immunise human subjects with the analogue, and examine whether an immune response is elicited. For this sort of study, it is not possible to use human subjects, so rabbits were deemed a suitable alternative experimental model. Although human transferrin is antigenic in rabbits, there is a high degree of sequence and predicted secondary structure homology between rabbit transferrin and HST (see [3]). Therefore, a rabbit immunisation study would demonstrate whether or not the HIV-1 peptide cleavage site sequence in the context of an HST analogue is strongly immunogenic or not.

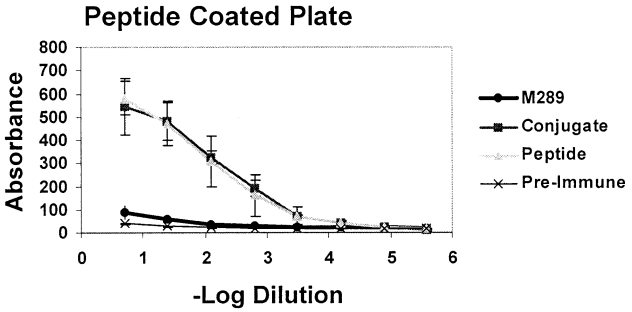

Fig. 1. The HIV-1 protease cleavage site in the M289 analogue is not immunogenic in rabbits. Rabbits were immunised three times with the M289 analogue, a conjugate of the recombinant wild-type HST and HIV-1 protease cleavage site peptide, or the peptide alone. The amounts of antibody specific for the HIV-1 protease cleavage site were assayed using an antibody capture ELISA. Pre-immune serum was analysed as a control. Immune serum samples were analysed in triplicate. The results shown here are the mean ELISA results (absorbance at 695 nm) for all rabbits immunised with a particular antigen. For pre-immune serum, the value plotted is the mean of single ELISA readings of serum from each rabbit. Error bars indicate one standard deviation of the mean.

Experimentally, rabbits were immunised with the recombinant M289 analogue protein, and control animals immunised with either the HIV-1 protease cleavage site peptide, or a chemical conjugate of the peptide and recombinant w.t. HST. The amounts of antibody specific for the HIV-1 protease cleavage site peptide produced by these animals were determined using a standard antibody capture assay with antigen-coated plates.

Strong immune responses were elicited against the HIV-1 protease cleavage site peptide in all animals immunised with either the peptide or the HST/peptide conjugate (Fig. 1). However, the M289 analogue induced only a very poor immune response, barely measurable above background levels.

It could be argued that failure of the foreign peptide in the M289 analogue to elicit an immune response could be an

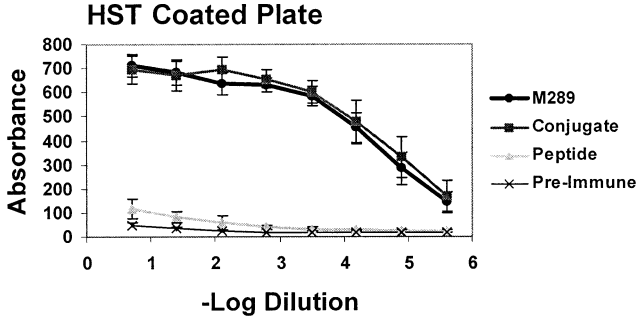

Fig. 2. Determination of HST specific antibodies. The same samples as described in Fig. 1 were used to determine the amounts of antibody specific for HST. Pre-immune serum was analysed as a control. Immune serum samples were analysed in triplicate. The results shown here are the mean ELISA results (absorbance at 695 nm) for all rabbits immunised with a particular antigen. For pre-immune serum, the value plotted is the mean of single ELISA readings of serum from each rabbit. Error bars indicate one standard deviation of the mean.

experimental artefact caused by an inadequate immunisation procedure, or some unknown immunological inhibitor present in the protein preparation. One way to show that an immune response was mounted in these animals would be to demonstrate the presence of antibodies reactive against the HST region of the M289 analogue. The M289 analogue should induce an equivalent titre of anti-HST antibody as the rabbits immunised with the chemical conjugate. The results of this experiment are shown in Fig. 2. Animals immunised with either M289 or the peptide/HST conjugate produced equivalent high titres of anti-HST antibodies. No anti-HST antibodies were detected in serum from animals immunised with the peptide alone. This shows that the rabbits were correctly immunised and were capable of a productive immune response.

4. Discussion

Recently, we proposed a novel approach for the biological delivery of peptide drugs could be to incorporate the sequence of the peptide into the structure of a natural transport protein such as HST [3]. We demonstrated that this was indeed possible, by inserting a sequence cleaved by HIV-1 protease into a surface exposed loop of HST. One concern raised over these HST analogues was the possibility that the HST could enhance the immunoreactivity of the peptide, in the same way that carrier proteins can be used to generate highly immunogenic peptide hapten conjugates. In this study, we conducted rabbit immunisation studies to determine the immunogenicity of the peptide moiety.

Our studies demonstrate strong immune responses were elicited against the HIV-1 protease cleavage site peptide in all animals immunised with either the peptide or the HST/peptide conjugate. However, the M289 analogue induced only a very poor immune response, barely measurable above background levels. It could be argued that failure to produce an immune response against the foreign peptide in the M289 is because the immune response triggered by HST itself could mask the antigenicity of the peptide sequence. However, data from two other studies demonstrate that it is possible to produce strong immune responses to peptide antigens engineered into very immunogenic carrier proteins. In the first, which examined the immunogenicity of foreign peptides engineered into poliovirus antigen chimeras [6], defined regions of the CDR2-like region of the T cell antigen CD4 were inserted into the antigenic site 1 of Sabin type 1 poliovirus. Serum from rabbits immunised with the recombinant virus was strongly immunoreactive against both poliovirus, the T cell antigen and soluble CD4. This was also the case in a second study, which looked at the immunogenicity of an HIV-1 neutralising epitope introduced into a surface exposed region of the highly immunogenic cholera toxin B subunit [7].

In the absence of any further data, it would be unwise to speculate upon a possible mechanism for the immunogenic attenuation observed in this study. It is known that there are differences in processing and presentation of peptides, peptide-transferrin conjugates and recombinant proteins by cells of the immune system (see [8–12]), but clearly this requires further investigation. This study is an extension of earlier work to develop alternative means for delivery of peptide drugs [3,5,13–16]. The new data provided here will be beneficial not only for our future goals, but could also have an important impact on the future design of peptide delivery systems.

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