SPECIFIC BINDING MEMBERS AGAINST SYNAPTOPHYSIN

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Liver fibrosis is a reversible process characterised by an accumulation of extracellular matrix protein in the liver that precedes the development of cirrhosis and liver failure (Friedman S.L. J Biol Chem 2000;275:2247-50; Bataller R. et al. Semin Liver Dis 2001;21:437-51). A number of conditions can cause damage to the liver resulting in fibrosis, including viral infections (e.g. Hepatitis C) and alcohol misuse. Fibrosis can remain undetected for many years and can inflict severe damage that is sometimes fatal. Despite a global population of in excess of potentially 200 million people suffering from liver fibrosis, there are no therapeutic options available to clinicians to treat this condition.

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majority of extracellular matrix proteins that constitute the scarring observed in liver fibrosis. There is strong evidence to suggest that activated HSCs and fibrogenesis are deleterious responses to chronic liver damage of any cause and that increased activated HSC apoptosis can resolve fibrosis and enhance the liver's response to chronic damage (Iredale J.P. et al. J Clin Invest 1998;102:538-49; Wright M.C. et al. Gastroenterology 2001;121:685-98; Orr J.G. et al. Hepatology 2004;40:232-42; Issa R. et al. Gut 2001;48:548-57).

A major problem for many potential anti-fibrotics in the past is that the drugs did not reach therapeutic concentrations within hepatic stellate cells, possibly because of the proximity of hepatocytes, which function to metabolise exogenous compounds. These drugs include a number of agents that have anti-inflammatory activity *in vitro* and *in vivo* and which may reduce stellate cell activation. These include corticosteroids, antagonists to TNFα, anti-oxidants, cytokines (γ interferon) and hepatocytes growth factor (HGF) PPARγ ligands (thiazolidinediones), endothelin-1 antagonists, halofuginone (an anticoccidial drug) and gene therapy (administration of metalloproteinase mRNA via gene therapy in animal models). The lack of success indicates that it would be useful to target any anti-fibrotic therapies to hepatic stellate cells in the liver.

It has been shown recently by Polestra and colleagues that the mannose 6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptor is expressed at high levels in activated hepatic stellate cells during fibrosis and that serum albumin (SA) modified with mannose 6-phosphate (M6P) distributes to the liver when administered to rats (molar ratio of M6P:SA is 28:1) with 70% of the intra hepatic dose found in hepatic stellate cells (Beljaars et al. Hepatology 1999; 29: 1486-93). SA modified with 10 cyclic peptide moieties recognizing

collagen type VI receptors (C*GRGDSPC*, in which C* denotes the cyclizing cysteine residues) also results in preferential distribution to the rat liver within 10 minutes after intravenous injection (Beljaars et al. J Biol Chem 2000; 275: 12743-51). In fibrotic livers 70% of the hepatic dose of the peptide-modified albumin was associated with activated hepatic stellate cells. However, in human liver tissue perfusions, these reagents were taken up by kupffer cells, the hepatic cell type involved in the removal of large molecular weight molecules and foreign particles, and not stellate cells.

Synaptophysin is a protein expressed in neural cells and hepatic stellate cells only (Cassiman D. et al. Am J Pathol 1999;155:1831-1839; Bargou R.C. et al. Gene 1991;99:197-204). It is a membrane bound protein and is not available in a functional purified form. Despite this significant limitation the applicants have isolated successfully the first fully human monoclonal antibody fragment with specificity for an extracellular domain of synaptophysin, present on hepatic stellate cells. This antibody was isolated using the technique of phage display and a human antibody library made available by the MRC, Cambridge, UK. The antibody was raised against a peptide and it is this anti-peptide antibody that also recognises the whole native synaptophysin protein in its natural confirmation in the stellate cell membrane. Antibodies which recognise and bind to synaptophysin have the potential to provide, for the first time, a reagent suitable for a number of therapeutic applications as discussed below.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Schematic diagram of synaptophysin. Human synaptophysin has a theoretical molecular mass of 33.8kDa and is predicted to span the plasma membrane of cells as outlined. The protein is

reported to be glycosylated and experimentally has a molecular mass of ~38-40kDa (Eastwood S.L. et al. Brain Res Bull 2001;55:569-78).

Figure 2

Isolation and amplification of phage-antibodies (polyclonal) with affinity for the target peptide-BSA. The enrichment of phage-antibodies (polyclonal) recognising the target peptide-BSA antigen during bio-panning was assessed by ELISA. Polyclonal phage-antibodies (~$1x10^{10}$) rescued from each round of selection were assayed for binding to the target peptide-BSA conjugate. Bound phage were detected using HRP-labelled anti-M13 antibody (Pharmacia) as outlined in methods section. Phage titre at each pan is indicated (---□---).

Figure 3

C1-phage antibody clone (monoclonal) assayed for binding to the target peptide-BSA as outlined for Figure 2.

Figure 4

Nucleotide and amino acid sequence of the C1-3 scAb within the pIMS147 vector (alignment of SEQ ID NO 7 and SEQ ID NO 8). The hypervariable complementarity determining regions (CDRs), which make up the antigen binding site, are in bold. The flexible amino acid linker $(Gly_4,Ser)_3$ that joins the H and L chains is underlined (_____). The start of the Framework regions (FW) and the start of the Human constant kappa domain (C_K) are also indicated. Cloning sites are underlined (__)with the corresponding restriction enzyme indicated above the amino acid sequence. The beginning of the HuCk constant domain and 6 Histidine residue purification tag is shown - full amino acid sequence:

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Figure 5

Induction of C1-3 scAb expression in XL-1 blue cells and purification by Ni affinity chromatography. Upper blot, coomassie blue stained samples after SDS-PAGE with protein loadings as indicated; lower blot, Western analysis of samples with anti-human Cκ light chain antibody.

Figure 6

Anti-human Cκ light chain capture ELISA quantitation of C1-3 scAb. Goat anti-human Cκ light chain antibody was coated onto the surface of a ninty six well plate. Dilutions of human IgG or C1-3 scAb preparation were incubated in individual wells. After extensive washing, captured IgG (-□-) or scAb (-o-) was quantitated as outlined in the methods section. Data are the mean and standard deviation of 3 separate determinations - typical of 7 separate preparations.

Figure 7

Expressed scAb was tested for its ability to interact with the peptide YPFRLHQVYFDAPSC (SEQ ID NO: 9) by ELISA using anti-BSA-target peptide scAb. Wells of a 96 well plate were coated with BSA-target peptide or BSA and incubated with either C1-3 scAb (grey bars) or control scAb incubation buffer (clear bars) followed by extensive washing. Bound scAb was detected using the HRP conjugated anti-human Cκ light chain antibody and quantitated as outlined in methods section. Data are the mean and standard deviation of 3 separate determinations. 'BSA-peptide 2' is the target peptide.

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Western blot showing the binding of C1-3 scAb to the target peptide. Cell extracts (5µg/lane) were subjected to Western blotting and membranes probed with C1-3 scAb followed by incubation with HRP conjugated anti-human Cκ light chain

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Figure 9

C1-3 scFv was labelled with FITC and confirmed by SDS-PAGE. Ni^{2+} charged IMAC Fast Flow Sepharose resin purified C1-3 acAb was subjected to dialysis and then concentrated using a centricon YM-3 centrifugal concentrator. Concentrated C1-3 scAb was then FITC-labelled and aliquots of each fraction (approx 0.1µg/lane) subjected to SDS-PAGE followed by coomassie blue (total protein) staining of gel.

Figure 10

FACS analysis of human HSC incubated without (UPPER PANEL) or with (LOWER PANEL) primary antibodies - i.e. FITC-C13 scAb (FITC) and a mouse monoclonal antibody to α-smooth muscle actin (αsma-APC). Anti-α-smooth muscle actin antibody was detected using a biotin conjugated secondary antibody followed by fluorophore-streptavidin conjugate incubation as outlined in the Methods section. Figures represent the percentage of cells in each quadrant. Data are typical of 3 separate cell preparations.

Figure 11

Uptake of C1-3 scAb and C1-3-conjugates by human HSCs in culture. HSCs seeded into 24 well plates were incubated in 0.3mls culture medium containing 5µg scAb. Samples of medium were taken at the indicated times and subjected to Western blotting to detect scAb using HRP-conjugated anti-human Cκ light chain antibody (UPPER PANEL) and serum albumin (LOWER

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