Arab Journal of Gastroenterology 10 (2010) S3–S6

Contents lists available at ScienceDirect

Arab Journal of Gastroenterology

journal homepage: www.elsevier.com/locate/ajg

Review

Using a recombinant single chain antibody for targeting liver myofibroblasts with anti-fibrogenic therapeutics

Angela Douglass [a,b,c], Karen Wallace [a], Matthew Koruth [c], Caroline Barelle [b], Andrew J. Porter [c], Matthew C. Wright [a,*]

[a] Institute of Cellular Medicine, University of Newcastle, UK
[b] Wyeth Research, Polwarth Building, Foresterhill, Aberdeen, UK
[c] School of Medical Sciences, University of Aberdeen, UK

ARTICLE INFO

Keywords:
C1-3
Gliotoxin
Hepatic stellate cell
Fibrosis
Kupffer cell
Matrix metalloproteinase 13

ABSTRACT

Chronic liver disease results in a liver scarring response termed fibrosis. Excessive scarring leads to cirrhosis which is associated with high morbidity and mortality. The only treatment for liver cirrhosis is liver transplantation, therefore much attention has been directed toward therapies that will slow or reverse fibrosis. Although anti-fibrogenic therapeutics have been shown to be effective in experimental animal models, licensed therapies have yet to emerge. A potential problem for any anti-fibrogenic therapy in the liver is the existence of the body's major drug metabolising cell (the hepatocyte) adjacent to the primary fibrosis-causing cell, the myofibroblast. A human recombinant single chain antibody that binds to the surface of myofibroblasts was therefore developed. This antibody bound specifically to myofibroblasts in fibrotic mouse livers. When conjugated with a compound that stimulates myofibroblast apoptosis, the antibody directed the specific apoptosis of myofibroblasts with greater specificity and efficacy than the free compound. The antibody also reduced the side-effect of liver macrophage apoptosis and – in contrast to the free compound – reversed fibrosis in the sustained injury model used. These data suggest that specifically stimulating the apoptosis of liver myofibroblasts using a targeting antibody has potential in the treatment of liver fibrosis.

Introduction

Fibrosis is a progressive disease characterised by the accumulation of scarring extracellular matrix proteins which disrupt normal tissue architecture. As fibrosis worsens, tissue function is impaired and in vital organs such as the liver – in the absence of transplantation – morbidity and mortality result [1]. A pragmatic approach to the treatment of chronic disease is the alleviation of fibrosis. Previous work has demonstrated that the liver myofibroblast (derived from hepatic stellate cells or other sources) is primarily responsible for fibrogenesis in the liver and that experimental therapies that stimulate liver myofibroblast apoptosis reverse fibrosis and improve liver function in animal models [2–5].

However, directing cell death as a mode of treatment for a disease comes with risk because most therapeutics are unlikely to act specifically on the target cells alone. The risk is therefore the stimulation of cell death in cells other than the target cells, with potential adverse effects.

To increase the specificity of action of a potential therapeutic to stimulate liver myofibroblast death, a human recombinant single chain antibody (scAb) was developed (termed C1-3 [6]) to an extracellularly-exposed region of synaptophysin, a plasma membrane protein expressed in the liver specifically in human and rodent myofibroblasts [7]. Synaptophysin is primarily associated with neural tissue and is thought to be involved in synaptic vesicle exo-endocytosis. However, synaptophysin knock out mice show no phenotype or detectable effects on synaptic transmission [8]. A potential advantage of using synaptophysin as a target to deliver therapeutics is that this protein – at least in neural tissue – forms part of an endocytosing vesicle, which may increase chances of scAb and conjugated therapeutic uptake.

Phage display

Phage display is a powerful technique for selecting and cloning sequences that encode proteins with a native affinity for a particular ligand. In the case of C1-3, a phage library was employed in which potentially the entire human repertoire of variable heavy

* Corresponding author. Address: Institute of Cellular Medicine, School of Clinical and Laboratory Sciences, Level 2 William Leech Building, Medical School, Framlington Place, University of Newcastle, Upon Tyne NE2 4HH, UK.
E-mail address: M.C.Wright@ncl.ac.uk (M.C. Wright).

1687-1979/$ - see front matter © 2010 *Arab Journal of Gastroenterology*. Published by Elsevier B.V.
doi:10.1016/j.ajg.2009.12.012

and variable light chains (which encode antibody antigen binding sites) were cloned as a single fused domain into the gIII M13 bacteriophage coat protein (Fig. 1). The phage "display" different variable domains on their surface, thus generating a phage display library. Variable domains which interact with a particular ligand can be selected by a process of "panning", whereby phage are exposed to an immobilized target sequence. Unbound phage are removed by washing and bound phage eluted and amplified through re-infection of *Escherichia coli* (Fig. 1) The process of panning is repeated several times allowing for isolation of specific high-affinity antigen binding phage. Single clones are then isolated and analysed prior to the sub-cloning the scAb-encoding region into an expression vector. Using established procedures such as protein tagging, high levels of pure monoclonal scAb can be generated with relative ease.

C1-3 scAb

A range of human recombinant scAbs were generated to synaptophysin by phage display. One of these – C1-3 – was selected and its ability to act as a targeting agent for liver myofibroblasts examined. Fluorescently-labelled C1-3 scAb avidly bound to myofibroblasts (see Fig. 2) but not hepatocytes [6]. The C1-3 was taken up into myofibroblasts by pinocytosis [6]. The C1-3 scAb alone was not toxic to myofibroblasts *in vitro* but when conjugated with tributyl tin, the scAb directed the toxin to myofibroblasts (with toxin activity retained) [6].

To determine whether the C1-3 scAb could be effective as a drug targeting agent *in vivo*, mice with liver fibrosis (via carbon tetrachloride administration) were injected *i.p.* with C1-3 scAb. Initial studies used fluorescently-labelled C1-3 to examine its distribution throughout the body up to 24 h after injection. The results of these studies showed that the C1-3 scAb appeared in the serum within 20 min and was eliminated with an approximately half-life of 2 h [9]. The C1-3 scAb was detectable in liver homogenate but undetectable in brain, muscle or spleen [9]. Immunohistochemical analysis indicated that the C1-3 scAb localised to the liver, in regions where scars were present. There was minimal immunohistochemical evidence for the presence of C1-3 scAb in non-fibrotic liver [9]. Co-staining of fibrotic liver sections showed that the C1-3 scAb co-localised with myofibroblast α-smooth muscle actin, but not the monocyte and macrophage marker F4/80 [9]. These data indicate that the C1-3 scAb readily and selectively targeted liver myofibroblasts in an animal model of liver fibrosis.

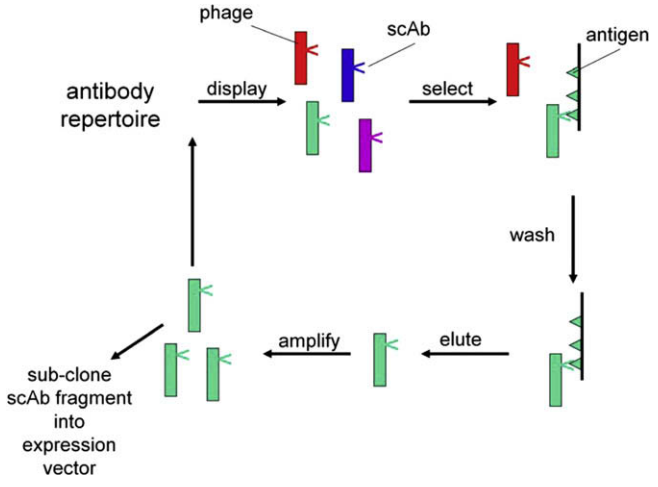

Fig. 1. A, schematic diagram of recombinant M13 bacteriophage incorporating a scAb within its coat protein and the procedure of phage display.

To determine whether C1-3 could deliver a functional experimental anti-fibrogenic therapeutic to myofibroblasts, C1-3 protein was chemically conjugated with gliotoxin. Free gliotoxin has been shown in previous work to stimulate the apoptosis of liver myofibroblasts *in vitro* [10] and *in vivo* [11] and to enhance recovery from liver fibrosis *in vivo* [2,11,5]. However, gliotoxin also causes – although to a lesser degree – the apoptosis of hepatocytes and stimulates Kupffer cell death [4,12].

Gliotoxin was chemically conjugated to either a C1-3 scAb or a control CSBD9 scAb (selected for its ability to bind to an irrelevant ligand) using *N*-(*p*-maleimidophenyl) isocyanate and *S*-acetyl thioglycolic acid *N*-hydroxysuccinimide [9]. Conjugation did not significantly alter scAb affinities for their respective antigens (as determined using antigen ELISA and BIAcore [9]) or ablate gliotoxin's ability to cause liver myofibroblasts apoptosis (see Fig. 3).

Mice were administered carbon tetrachloride twice weekly for eight weeks to generate liver fibrosis. During the last week prior to the final injection of carbon tetrachloride, mice were treated with a single dose of C1-3 scAb or C1-3-gliotoxin scAb. The mice were therefore treated with a potential therapeutic and injury was sustained by further treatment with carbon tetrachloride. Fibrotic mice were also treated with an equivalent dose of free gliotoxin or gliotoxin conjugated to CSBD9 (i.e., CSBD9-GT).

The results are summarised in Table 1 and show that free gliotoxin reduced the number of myofibroblasts but did not reduce fibrosis severity in the liver. C1-3-gliotoxin more potently reduced the number of myofibroblasts present compared to the same dose of free gliotoxin and additionally significantly reduced the severity of fibrosis in the liver. C1-3 scAb, CSBD9 scAb and CSBD9-gliotoxin had no effect on the number of liver myofibroblasts or fibrosis severity. Interestingly, free gliotoxin reduced the number of F4/80 positive cells (i.e., monocytes and macrophages/Kupffer cells) and MMP-13 levels in the liver whereas C1-3-gliotoxin did not. Macrophage depletion abrogates recovery from liver fibrosis [13]. Retaining macrophages in C1-3-gliotoxin treated animals may account for the improved anti-fibrogenic effect of gliotoxin when targeted to liver myofibroblasts with C1-3.

Discussion

Two major hurdles must be overcome to advance successful treatments for fibrosis. The first is the effective assessment of fibrosis in both experimental and clinical studies. It remains problematic to accurately screen for fibrosis using a battery of serum markers, particularly for those at early and intermediate stages of disease [14]. Biopsy remains the "gold standard" diagnostic tool for liver disease [15] but has significant drawbacks (the procedure is risky; repeated biopsy is inadvisable; and it does not give a globally accurate picture of organ fibrosis). The second hurdle is specificity and efficacy of potential treatments (in the liver – the myofibroblasts lie adjacent to the major drug metabolising cell of the body – the hepatocyte).

This laboratory has examined the potential of an antibody-based approach to tackle both imaging and therapeutic delivery in fibrosis. Although the C1-3 scAb does not directly bind to fibrosis proteins (it binds to the major fibrogenic cell), an image-visible scAb derivative may be effective in providing an indication of fibrosis severity. If successful as an imaging agent, experimental and clinical imaging studies should aid in its development as a targeting agent for anti-fibrogenic therapeutics.

The utility, or therapeutic efficacy of whole antibody, or related antibody structures, *in vivo* is influenced by a number of competing factors. These include: specificity, affinity (including avidity) and serum half-life. The C1-3 has already been expressed successfully as a monomeric scAb (38 kDA). In addition, it could also be used