Research Article

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A new fluorescence-based optical imaging method to non-invasively monitor hepatic myofibroblasts *in vivo*

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Background & Aims: Currently, staging of fibrosis in preclinical rodent liver fibrosis models is achieved histologically. Many animals are used at multiple time-points to assess disease progression or therapeutic responses. Hepatic myofibroblasts promote liver fibrosis therefore quantifying these cells *in vivo* could assess disease or predict therapeutic responses in mice. We fluorescently labelled a single chain antibody (C1-3) that binds hepatic myofibroblasts to monitor fibrogenesis *in vivo*.

Methods: CCl_4 was used to induce acute liver injury in WT and *cRel*$^{-/-}$ mice. Bile duct ligation was used to model chronic fibrosis. Hepatic myofibroblasts were depleted using a liposome-drug delivery system or chemically with sulfasalazine. An IVIS® spectrum visualised fluorophore-conjugated C1-3 *in vivo*.

Results: IVIS detection of fluorescently labelled-C1-3 but not a control antibody discriminates between fibrotic and non-fibrotic liver in acute and chronic liver fibrosis models. *cRel*$^{-/-}$ mice have a fibro-protective phenotype and IVIS signal is reduced in CCl_4 injured *cRel*$^{-/-}$ mice compared to wild-type. *In vivo* imaging of fluorescently labelled-C1-3 successfully predicts reductions in hepatic myofibroblast numbers in fibrotic liver disease in response to therapy.

Conclusions: We report a novel fluorescence imaging method to assess murine hepatic myofibroblast numbers *in vivo* during liver fibrosis and after therapy. We also describe a novel liposomal antibody targeting system to selectively deliver drugs to hepatic myofibroblasts *in vivo*. C1-3 binds human hepatic myofibroblast therefore imaging labelled-C1-3 could be used for clinical studies in man to help stage fibrosis, demonstrate efficacy of drugs that promote hepatic myofibroblast clearance or predict early therapeutic responses.

Lay summary: In response to damage and injury scars develop in the liver and the main cell that makes the scar tissue is the hepatic myofibroblast (HM). C1-3 is an antibody fragment that binds to the scar forming HM. We have fluorescently labelled C1-3 and given it to mice that have either normal or scarred livers (which contain HM) and then used a machine called an *in vivo* imaging system (IVIS) that takes pictures of different wavelengths of light, to visualise the antibody binding to HM inside the living mouse. Using fluorescently labelled C1-3 we can assess HM numbers in the injured liver and monitor response to therapy. We have also used C1-3 to target drugs encapsulated in lipid carriers (liposomes) to the HM to kill the HM and reduce the liver disease.

Keywords: Non-invasive imaging; Hepatic myofibroblasts; Fibrosis; Cell-targeting; Mouse.

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Abbreviations: HM, hepatic myofibroblast; αSMA, alpha-smooth muscle actin; WT, wild-type; ScAb, single chain antibody; CCl_4, chronic carbon tetrachloride; NIR, near infra-red; ROI, region of interest; ALT, alanine transaminase; AST, aspartate aminotransferase; PBS, phosphate buffered saline; BDL, bile duct ligation; ALP, alkaline phosphatase; DOX, Doxorubin; SYN, synaptophysin.

Introduction

Liver fibrosis is characterised by excess deposition of collagens by the primary scar-producing cell in the liver the hepatic myofibroblast (HM) [1]. HM are generated from quiescent hepatic stellate cells (qHSC) upon injury via a process called activation [2]. In acute liver injury HM produce a temporary scar to allow wound healing and once the liver has regenerated and normal homeostasis is restored, the HM are cleared by apoptosis or undergo de-differentiation [3]. In chronic injury HM persist, migrate and proliferate promoting scar formation and fibrosis [4,5]. Liver fibrosis is a highly dynamic process that can either progress or resolve. The HM is a key cell type regulating the kinetics of fibrosis and fibrolysis. HM accumulate during progression of liver fibrosis but their clearance precedes fibrolysis and remodeling of the scar matrix [6–8]. If a drug successfully treats the underlying cause of injury, promotes HM apoptosis or if the injury stimulus is removed the scar is remodeled [6,9].

Currently, histologically assessing fibrosis (Sirius Red) and HM numbers (alpha-smooth muscle actin (αSMA)) in the liver is the only accurate method to stage fibrosis in murine preclinical liver fibrosis [5,10,11]. Therefore large numbers of animals are used for multiple time-points to monitor disease kinetics or test novel anti-fibrotic drugs. Developing a method to image and assess HM *in vivo* would allow researchers to perform minimally invasive longitudinal monitoring of fibrosis progression or resolution. This could reduce the number of mice required to perform liver

disease models when comparing wild-type (WT) and transgenic mice or predict early therapeutic responses when testing anti-fibrotic drugs.

Synaptophysin mRNA is detected in rat quiescent hepatic stellate cells (qHSC) and cultured HM. Immuno-histochemical staining demonstrated co-localisation of synaptophysin and αSMA in fibrotic liver [12]. C1-3-GT has previously been used to deplete HM from the livers of acute and chronic carbon tetrachloride (CCl_4) injured mice [13], proving that C1-3 binds and successfully delivers drugs to HM *in vivo*. However, this drug targeting approach is limited by suitability of the drug in conjugation chemistry. This study aims to exploit the HM binding properties of C1-3 to develop two novel tools for fibrosis research. Firstly, develop a novel imaging probe to monitor HM *in vivo*. Secondly, conjugate C1-3 to liposomal carriers to selectively deliver drugs to HM *in vivo*.

Materials and methods

C1-3 Production and purification

pIMS-147-C1-3 plasmid transformed E-Coli XL-1 blue cells were cultured in Lysogeny broth media containing antibiotics (ampicillin and tetracycline). E-Coli expressing pIMS-147-C1-3 were cultured in Luria-Bertani broth overnight then in Terrific Broth media for 8 h. C1-3 expression was induced by adding isopropyl β-D-1-thiogalactopyranoside to culture media for 4 h. Cells were lysed and C1-3 was purified from the supernatant using immobilised metal affinity chromatography as previously described [13–15]. Endotoxin was removed using Q maxi H columns (Sartorious Vivascience).

Liposomal preparation

C1-3 or CSBD9 coated sterically stabilised liposomes loaded with doxorubicin were prepared as previously described [16]. Full details in Supplementary materials and methods.

Mice and models of liver injury

All experiments were performed on male C57BL/6 (WT) or global *cRel* knockout mice ($cRel^{-/-}$) mice under approval from the Newcastle Ethical Review Committee and a UK Home Office licence. Acute liver damage was induced by intraperitoneal injection of a single of dose of CCl_4 at 2 μl/g body weight (CCl_4:olive oil at 1:1 [vol/vol]). Bile duct ligation (BDL) was achieved by surgically exposing the common bile duct followed by its double ligation. Appropriate pain relief was given to all mice. Mice developed chronic liver fibrosis for a period of 14 days [17].

Therapies

At 24 h post CCl_4 injury animals were intravenously injected with C1-3 or CSBD9 coated sterically stabilised liposomes loaded with 5 mg/kg doxorubicin or empty liposomes. Free doxorubicin was given at a concentration of 5 mg/kg. Chemical depletion of HM was achieved by intraperitoneal injection of a single of dose sulfasalazine (150 mg/kg) 24 h post injury.

In vivo fluorescent imaging

Conjugation of C1-3 or CSBD9 to DyLight800 (Thermo Scientific) fluorophore was performed following manufactures instruction. Sufficient quantities of C1-3 was produced and labelled prior to each individual experiment to control for antibody batch-to-batch variation or differences in labelling efficiency. Mice under isoflurane were fluorescently imaged (745/800 em/ex filters) using epi-fluorescence on an IVIS spectrum (Caliper Life Sciences) at 2 h, 4 h and 6 h post *i.v.* injection of Dylight800 labelled-C1-3 for biodistribution studies. For all other experiments the *in vivo* imaging of fluorescent labelled-C1-3 or CSBD9 was performed at 6 h post C1-3/CSBD9 (10 mg/kg) administration. After the final scan mice were humanely killed and the liver, kidney and spleen were excised and IVIS imaged (745/800 em/ex filters). Images were analysed using Living Image 4.3.1 software, regions of interest (ROI) were drawn as described in the Supplementary methods section and average radiant efficiency [p/s/cm^2/sr]/[μW/cm^2] was calculated after subtracting the background signal.

Statistical analysis

Data was analysed using Excel or GraphPad Prism, p values were calculated using a two-tailed unpaired Student t test or one-way ANOVA with Newman-Kuels post hoc test and p <0.05 (*), p <0.01 (**) or p <0.001 (***) was considered significant.

Results

We show that synaptophysin expression is increased in culture-activated and *in vivo* activated mouse HM compared to qHSC (Supplementary Fig. 1A–C). The single chain antibody (ScAb) C1-3 recognises an extracellular domain in synaptophysin, a protein expressed on HM in the liver. C1-3 has been reported to bind to HM but not qHSC or other liver cell types [12,14,15]. We show binding of C1-3 to mouse and human HM in culture (Supplementary Fig. 1D) and report that the pro-apoptotic molecule gliotoxin (GT) induces death (reduced cell attachment) of both quiescent HSC and HM (Supplementary Fig. 1E–F). However, when GT is conjugated to C1-3 (C1-3-GT) this complex is ~25 fold less potent at inducing death of qHSC (C) than HM (D), suggesting that C1-3 efficiently targets and kills HM.

CCl_4 metabolism by the liver produces hepatotoxic free radicals [14]. Administration of one dose of CCl_4 to mice promotes hepatocyte death and induces an inflammatory response, which drives HM activation [18,19]. HM numbers peak between 48-72 h with the majority of HM then cleared from the liver by either apoptosis or de-differentiation by day 5 [20–22].

We wanted to establish the biodistribution and clearance kinetics of C1-3 labelled with a near infra-red (NIR) fluorescent dye (DyLight800) in mice with acute liver injury. 48 h acute CCl_4 injured WT C57/Bl6 mice were administered NIR fluorophore-conjugated-C1-3 via the tail-vein followed by whole body IVIS imaging at 2, 4, 6, 12 h, and 1 day. *In vivo* fluorescence signal peaks at 2 h and then rapidly falls between 2-6 h. A further decrease occurs between 6-12 h with the signal plateauing between 12-24 h (Supplementary Fig. 2A). *Ex vivo* imaging of organs at 48 h post CCl_4 revealed that the fluorescence signal was highest in the liver and little or no signal was detected in the heart, spleen, pancreas or brain (Supplementary Fig. 2B). ScAb are cleared by the kidneys and excreted into the urine [14,15] therefore we expect to detect some fluorescence signal in the *ex vivo* imaged kidney (Supplementary Fig. 2C). From the biodistribution experiments we decided to image mice at 6 h for future studies for three reasons: i) The majority of unbound antibody has been excreted; ii) there is a strong signal from the liver; iii) this time-point has the least mouse-to-mouse variation in fluorescence signal (Supplementary Fig. 2A).

To verify that IVIS signal from fluorescently labelled C1-3 originates from the liver after injury we performed 2D fluorescence imaging (Supplementary Fig. 3A–B) and Fluorescent Imaging Tomography (FLIT) imaging (Fig. 1A), which uses geometry, depth and intensity of the signal to build a 3D reconstruction of the mouse and provide an anatomical localisation of the fluorescence signal. 3D FLIT images show retention of fluorescently labelled-C13 in the liver (Supplementary Movie 1), conversely, signal from fluorescently labelled-CSBD9, a control ScAb predominantly