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NF-κB is a critical regulator of the survival of rodent and human hepatic myofibroblasts☆

Martha R. Watson[1], Karen Wallace[1], Roben G. Gieling[1], Derek M. Manas[1], Ellis Jaffray[2], Ronald T. Hay[2], Derek A. Mann[1], Fiona Oakley[1,*]

[1]*Liver Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle NE2 4HH, UK*
[2]*Centre for Interdisciplinary Research, School of Life Science, University of Dundee, DD1 5EH, UK*

***Background/Aims*: Hepatic myofibroblast activation during injury causes deposition of extracellular matrix within the liver and promotes development of fibrosis. Hepatic myofibroblast apoptosis is associated with remodelling of fibrotic extracellular matrix and regression of fibrosis. Previous work showed that inhibition of constitutive NF-κB signaling promotes hepatic myofibroblast apoptosis and resolution of fibrosis in rodent models. However, to date agents used to target constitutive NF-κB transcriptional activity in hepatic myofibroblasts have been relatively non-specific with potential for off-target effects that may complicate data interpretation. Likewise, rat chronic liver disease models may not accurately recapitulate the activation of human hepatic myofibroblasts.**

***Methods*: We used a mutant recombinant IκBα super-repressor fused to the HIV–TAT domain to specifically target NF-κB signaling in hepatic myofibroblasts. Inhibition of NF-κB activity was measured using reporter assay. Apoptosis of hepatic myofibroblasts was assessed by morphological changes, cleavage of the PARP-1 protein and Caspase 3 activation.**

***Results*: TAT-IκBαSR reduced NF-κB dependent transcription, Bcl-2 expression and promoted Jun-N-terminal kinase-dependent apoptosis in human and rat hepatic myofibroblasts.**

***Conclusions*: These data highlight the conserved role of NF-κB during fibrogenesis. Our data validate the use of rodent models for pre-clinical testing of NF-κB inhibitors as anti-fibrotics and stimulators of fibrotic extracellular matrix remodelling.**

***Keywords*: NF-κB (NF-kappaB); Hepatic myofibroblast; Liver fibrosis; Apoptosis**

1. Introduction

Liver disease accounts for a high level of mortality worldwide. Fibrosis of the liver occurs alongside prolonged injury and its progression to end-stage cirrhosis is accompanied by a loss of organ function. The hepatic myofibroblast (HM), which is derived from the hepatic stellate cell (HSC), is a key mediator of this fibrotic process. Traditionally, liver fibrosis has been seen as an irreversible phenomenon, however recent studies demonstrate the potential for regression of fibrogenesis both in clinical and experimental models [1–3]. Regression of fibrosis is associated with apoptosis of HM which effectively removes the major cells responsible

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* Corresponding author. Tel.: +44 191 222 3852.
E-mail address: fiona.oakley@ncl.ac.uk (F. Oakley).

Abbreviations: Bcl-2, B-cell CLL/lymphoma 2; DNA, deoxyribonucleic acid; EMSA, electrophoretic mobility shift assay; HM, hepatic myofibroblast; HSC, hepatic stellate cell; IKK, IκBα kinase; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; TIMP-1, tissue inhibitor of metalloproteinase 1.

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for production and maintenance of fibrotic matrix [4,5]. HM display constitutive activation of the NF-κB transcription factor. An important pro-fibrogenic function of NF-κB in HM is stimulation of Gadd45β and suppression of Jun N-terminal kinase (JNK) mediated apoptosis [6]. Consequently, agents targeting the NF-κB transcriptional cascade, such as gliotoxin, sulphasalazine and thalidomide, induce HM apoptosis and promote regression of liver fibrosis in rodent models [5–7].

Most studies on HM biology are derived from an *in vitro* model in which purified primary rodent or human HSC are cultured on plastic and over several days are transdifferentiated to the myofibroblastic phenotype. It has recently been reported that *in vitro* generation of the fully mature HM phenotype requires passage of human cells at least 4 times and that these myofibroblasts are significantly more resistant to apoptosis than their rodent counterparts. This has led to criticism of studies utilising rodent HM and doubts concerning the validity of data generated with rat models of fibrotic regression [8,9]. Similarly, gliotoxin, sulphasalazine and thalidomide have many additional targets outside the NF-κB signaling system [10–12]. There is hence understandable concern that NF-κB may not be a suitable target for promoting the regression of fibrosis in human liver disease. We have therefore tackled this debate by using a highly specific molecular inhibitor of NF-κB to determine a role for this transcription factor as a regulator of human HM survival.

The prominent form of NF-κB in HM is the classic p65:p50 heterodimer. In the majority of mammalian cells this heterodimer is sequestered within the cytoplasm *via* interaction with IκBα. Many of the signaling pathways that activate NF-κB transactivation converge at the IκB kinase (IKK) which phosphorylates two key residues within the IκBα protein, Serines 32 and 36 [13]. This phosphorylation targets IκBα for ubiquitination and proteasomal degradation, resulting in nuclear translocation of NF-κB and activation of target genes [14]. HM bypass this regulatory requirement for IκBα degradation since expression of this inhibitor is dramatically diminished during the transdifferentiation of HSC [14,15]. We therefore reasoned that experimental manipulation of IκBα protein expression in HM would selectively block NF-κB function, thus revealing any role for this transcription factor in apoptosis.

We show that fully mature human HM (passage 4+) display constitutive activation of NF-κB and expresses high levels of its downstream anti-apoptotic target genes Gadd45β and Bcl-2. We specifically inhibit NF-κB by using a recombinant cell-permeable IκBα super-repressor (IκBα SR, which is mutated at serine 32 and 36) protein and clearly demonstrate induction of JNK-dependent apoptosis. Together these data validate NF-κB as a putative therapeutic target for the treatment of human liver disease and support the continued use of rodent models of fibrosis in pre-clinical testing of drugs that target the activities of this transcription factor.

2. Materials and methods

2.1. Cell culture

Rat hepatic myofibroblasts (HM) were isolated from livers of Sprague–Dawley rats and cultured as previously described [16]. Similarly, human HM were isolated from livers of adult male patients after partial hepatectomy as approved by the UK South and West Local Research Ethics Committee and Newcastle and North Tyneside Local Research Ethics Committee and subject to patient consent. U937 cells stably transfected with a luciferase reporter plasmid containing 3 NF-κB consensus sequences inserted upstream of a minimal promoter to drive luciferase expression (U937-κB) were a kind gift from Professor Rune Blomhoff (University of Oslo, Norway) [17]. TAT-IκBα-S32A.S36A (SR) and TAT-GST proteins were produced as previously described [18] and used at 10–100 μg/ml. The Jun N-terminal kinase (JNK) inhibitor SP600125 and p53 inhibitor (pifithrin-α) were purchased from Calbiochem (UK).

2.2. SDS–PAGE and immunoblotting

Whole cell, nuclear and cytoplasmic protein extracts were isolated and quantified as previously described [16]. Western blotting was performed, as previously described using 20 μg of whole cell extract or 12 μg of nuclear or cytoplasmic extracts [15]. Primary antibodies: p50 NF-κB (Santa Cruz, 1:500), PARP-1 (Santa Cruz, 1:500), Bcl-2 (Abcam, 1:200), Gadd45β (Santa Cruz, 1:200), IκB-α Horseradish peroxidase-conjugated secondary antibodies; donkey anti-goat and goat anti-rabbit were purchased from Santa Cruz whilst Goat anti-mouse was purchased from Sigma and used at a dilution of 1:2000.

2.3. Plasmid DNA, cell transfections and reporter assays

Plasmid DNA was prepared using Maxiprep kit (Qiagen). The IκBα-Luc promoter and TIMP1-Luc promoter reporter gene constructs have been described elsewhere [6]. HM were transfected using the Effectene protocol (Qiagen) with 1 μg of reporter plasmid DNA and 10 ng of control Renilla plasmid pRLTK. After 24 h of transfection, HM were treated with TAT-GST or TAT- IκB-α SR for 16 h. U937-κB-Luc cells were plated at a density of 100,000 cells per/well, pre-treated for 30 min with either TAT-GST or TAT- IκB-α SR, then stimulated with LPS for 4 h. Reporter gene activity was determined using the dual-luciferase assay system (Promega).

2.4. Total RNA isolation and cDNA synthesis

Total RNA was isolated from 5×10^6 human or rat HM using the "Total RNA purification kit" (Qiagen) according to the manufacturers instructions. RNA was treated with 1 μl Dnase (Promega) for 30 min at 37 °C. First strand cDNA produced via incubation with 1 μl random hexamer primer ($p(dN)_6$) and RNase free water at 70 °C for 5 min. RNasin, 100 U Moloney Murine Leukemia Virus reverse transcriptase (MMLV), 1× MMLV buffer and 0.4 mM dNTP's (Promega) were added and the mix was incubated at 42 °C for 1 h.

2.5. Reverse transcriptase (RT)-PCR

PCR amplification of target genes were conducted using specific primers designed to recognise: Human Gadd45β Forward 5′-AAG TTGATGAATGTGGAC-3′ and Reverse 5′-CGGCTTTCTTCGCA GTCG-3′ (350 bp amplicon, 54 °C); Rat Bcl-2 Forward 5′-CAGAGG GCTACGAGTGG-3′ and Reverse 5′-CAGAGCGATGTTGTCCAC-