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Silencing tissue inhibitors of metalloproteinases (TIMPs) with short interfering RNA reveals a role for TIMP-1 in hepatic stellate cell proliferation

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ABSTRACT

Myofibroblastic, activated hepatic stellate cells (HSC) play a pivotal role in the development of liver fibrosis through the secretion of fibrillar collagens and the tissue inhibitors of metalloproteinase (TIMP)-1 and -2. TIMPs are believed to promote hepatic fibrosis by inhibiting both matrix degradation and apoptosis of HSC. In other cell types, there is evidence that TIMP-1 has effects on proliferation, however the role of TIMPs in the regulation of HSC proliferation remains unexplored. Therefore, we have used short interfering RNA (siRNA) to investigate the effects of autocrine TIMP-1 and -2 on HSC proliferation. TIMP-1 and -2 siRNA were highly effective, producing peak target protein knockdown compared to negative control siRNA of 92% and 63%, respectively. Specific silencing of TIMP-1, using siRNA, significantly reduced HSC proliferation. TIMP-1 was localised in part to the HSC nucleus and TIMP-1 siRNA resulted in loss of both cytoplasmic and nuclear TIMP-1. Attenuated proliferation was associated with reduced Akt phosphorylation and was partially rescued by addition of recombinant TIMP-1. We have revealed a novel autocrine mitogenic effect of TIMP-1 on HSC, which may involve Akt-dependent and specific nuclear mechanisms of action. We suggest that TIMP-1 might promote liver fibrosis by means other than its previously described anti-apoptotic effect on HSC. Moreover, these findings, together with our previous reports and the emerging data from *in vivo* studies of TIMP inhibition, provide strong evidence that TIMP-1 is mechanistically central to liver fibrosis and an important potential therapeutic target.

1. Introduction

Liver fibrosis represents the final common outcome of a wide range of chronic injurious processes in the liver. A wealth of evidence indicates that hepatic stellate cells (HSC) play a central effector role in the development of liver fibrogenesis. In response to liver injury and exposure to soluble mediators released from damaged hepatocytes and resident and recruited inflammatory cells, quiescent, vitamin A-storing HSC undergo a process of transdifferentiation to an activated phenotype [6,20]. Activated HSC contribute to the hepatic myofibroblast pool and express the principal components of the fibrotic neomatrix, including collagens type I and III [5]. HSC activation is accompanied by marked expansion in HSC number and expression of the tissue inhibitors of metalloproteinase (TIMP)-1 and -2 [10,13]. Activated HSC are the major source of TIMPs during chronic liver injury and therefore, in addition to producing the excess matrix that characterises fibrosis, HSC might shift the extracellular environment to one favouring matrix deposition [9,13].

TIMP-1 was originally named erythrocyte-potentiating activity for its ability to stimulate proliferation of erythroid progenitor cells [4,7] and modulates apoptosis and proliferation in a number of different cell types [3,27]. In cultured HSC, inhibition of extracellular TIMP-1 promotes apoptosis [17], which may result from reduced inhibition of MMPs and is likely to involve loss of survival signals from the extracellular matrix due to decreased availability of intact collagen I and enhanced cleavage of HSC surface proteins such as N-cadherin [8,31]. Therefore, manipulation of extracellular TIMP-1 appears to have significant effects on HSC survival and cell number. In support of this hypothesis, spontaneous recovery from liver fibrosis is associated with diminished TIMP expression and loss of HSC via apoptosis [9].

Proliferation of matrix-secreting HSC is also considered an important component pathway of liver fibrogenesis and one which may be targeted with anti-fibrotic effect [21,28]. A number of HSC mitogens have been identified, including platelet derived growth factor (PDGF) [16], vascular endothelial growth factor [29] and thrombin [15]. PDGF is a potent effector of HSC proliferation and the most studied, signalling via phosphatidyl inositol-3 kinase,

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Erk and other intracellular pathways [14,16,25]. Significantly, there is evidence showing that TIMP-1 has the ability to stimulate proliferation of fibroblasts in addition to haematoprogenitor cells [4,12]. Therefore, TIMPs might also promote HSC proliferation as an important component of fibrogenesis, in addition to their matrix-stabilising and anti-apoptotic effects. Evidence for such a role is currently lacking, so in this study we have used RNA interference to examine the effects of TIMP-1 and -2 on HSC proliferation.

2. Materials and methods

All reagents were obtained from Sigma–Aldrich Company (Poole, UK) unless otherwise stated.

2.1. Isolation of HSC

HSC were isolated from normal livers of ~300 g adult male Sprague–Dawley rats by sequential perfusion with collagenase and pronase, followed by discontinuous density centrifugation in 11.5% Optiprep. Activated HSC were generated by continuous culture of freshly isolated cells on plastic for 10–14 days, as previously described [18]. Passage 1 or 2 rat HSC were used for all experiments. Murine HSC were isolated using a modified version of the above protocol [11] and cultured under identical conditions as rat HSC.

2.2. TIMP-1 knockout mice

The murine TIMP-1 gene was targeted and mutant mice developed on a 129SV background as described by Soloway et al. [26]. Breeding pairs of mutant and wild-type (WT) mice, bred from heterozygotes, were shipped to the UK where their genotype was confirmed by PCR. Colonies of knockout (KO) and WT mice were established according to Home Office guidelines. Mice were used for experimental purposes after reaching adulthood (8 weeks).

2.3. Delivery of siRNA to HSC by electroporation

HSC at passage 1 were trypsinised and re-suspended in siPORT electroporation buffer (Ambion, Austin, TX, USA) prior to electroporation with siRNA using an ECM 830 square-wave electroporation system (Harvard Apparatus, Edenbridge, UK). All siRNA were purchased from Ambion. Briefly, 200 μl of 1×10^6 cells/ml suspension was mixed with 2.5 μl of 100 μM TIMP-1 or -2 siRNA, 2.5 μl of 100 μM negative control siRNA #1, or 2.5 μl nuclease-free H_20 as an electroporation control in 0.4 cm gap electroporation cuvettes. Electroporation was performed using a single 800 V 300 μs pulse and the cells immediately re-suspended in 37 °C pre-warmed culture medium to 2×10^5 cells/ml prior to plating into 24-well plates at a density of 1×10^5 cells/well, giving a final concentration of siRNA in culture of 25 nM. Untreated HSC served as an additional control. Cell-conditioned media were harvested at 24, 48, 72 and 192 h after electroporation, the culture medium having been replaced 24 h prior to each harvest. siRNA had the following sequences: Rat TIMP-1 siRNA sense 5′-CGGAAAUUUGCACAUCACU-3′; antisense 5′-AGUGAUGUGCAAAUUUCCG-3′. Rat TIMP-2 siRNA sense 5′-GGAAAGAAGGAAUAUCUAA-3′; antisense 5′-UUAGAUAU UCCUUCUUUCC-3′. Ambion Negative control siRNA #1 was used, which is a 19 bp scrambled sequence with no significant homology to any known rat gene. In certain experiments a second rat TIMP-1 siRNA (termed TIMP-1 siRNA 'B') was used with the following sequence: sense 5′-CCCACCCACAGACAGCUUU-3′; antisense 5′-AAA GCUGUCUGUGGGUGGG-3′.

2.4. Measurement of TIMP silencing by enzyme-linked immunosorbent assay and quantitative PCR

HSC conditioned media were assayed for TIMP-1 and -2 protein content using commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (TIMP-1, R&D Systems, Abingdon, UK; TIMP-2, GE Healthcare, Chalfont St. Giles, UK). TIMP content of each sample was determined using recombinant TIMP-1 or -2 standard curves after correction of any background reading. TIMP mRNA was assessed by reverse transcription real-time PCR of cell lysates collected 48 h after electroporation. Total RNA from cultured cells was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions and first strand cDNA synthesis was undertaken as previously described [18]. cDNA was stored at −20 °C or used immediately for polymerase chain reaction (PCR). For determination of TIMP-1 and -2 expression in rat samples, a reaction mixture was prepared consisting of 12.5 μl Taqman 2× Universal PCR master mix (Applied Biosystems, Warrington, UK), 0.3 μM of each primer, 0.3 μM of probe and 2 μl cDNA made up to a final volume of 25 μl with nuclease-free water. Samples were added to 96-well optical reaction plates. The PCR conditions were as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturing for 15 s at 95 °C and annealing and extension at 60 °C for 1 min. 18S ribosomal RNA and rat TIMP-2 Taqman™ primers and probe were purchased as proprietary gene expression assays from Applied Biosystems. Rat TIMP-1 primers and probe were purchased from Sigma–Genosys (Haverhill, UK) with the following previously reported sequences [18]: forward 5′-agcctg-tagctgtgccccaa-3′, reverse 5′-aactcctcgctgcggttctg-3′ and probe 5′-agaggctctccatggctgggggtgta-3′.

2.5. Effect of TIMP siRNA and rat TIMP-1 on cellular proliferation

Following electroporation with TIMP or negative control siRNA, or control treatment and culture for 48 h in 24-well plates as described above, HSC were pulsed with [^{3}H]thymidine (1 μCi per well) plus serum with or without recombinant rat TIMP-1 (rrTIMP-1, R&D Systems) 100 ng/ml for 18 h and thymidine incorporation was assessed as previously described [2]. In separate experiments, percentage change in cell number was determined by direct counting of HSC number performed at 24 and 72 h after siRNA treatment in six randomly chosen high power fields in duplicate wells for each condition. Proliferation of WT and TIMP-1 KO mouse HSC at passage 2 was also determined by [^{3}H]thymidine incorporation assay.

2.6. Immunofluorescence analysis of TIMP-1 silenced HSC

Following electroporation with TIMP or negative control siRNA, HSC were grown in glass slide chambers for 48 h and fixed using an ice-cold 1:1 mixture of methanol and acetone for 10 min at −20 °C. Slides were then air-dried at room-temperature and stored at −20 °C until immunostaining. HSC were immunostained using mouse anti-TIMP-1 monoclonal antibody (1:200; Millipore) or non-immune isotype control antibody, and FITC-conjugated rabbit anti-mouse IgG secondary antibody (1:100; Vector Laboratories, Peterborough, UK). Nuclei were counter-stained with Sytox Blue (Invitrogen). Immunofluorescence was detected by confocal microscopy and maximum projection images generated using optical slices captured exclusively through the cell nucleus.

2.7. Determination of Erk and Akt phosphorylation by Western blotting

Following electroporation with TIMP or negative control siRNA, or control treatment and culture for 48 h in 6 cm dishes, cells were