Regulation of matrix metalloproteinase-1 and α -smooth muscle actin expression by interleukin-1 α and tumour necrosis factor α in hepatic stellate cells

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Abstract Hepatic stellate cells (HSCs) are key players in liver fibrosis and regeneration via collagen degradation and synthesis. These phenomena involve inflammatory cytokines released from non-parenchymal liver cells such as Kupffer cells. Although the effects of individual cytokines on many cell types have been investigated in various conditions, such as inflammation and tissue fibrosis, investigating the effect of combined cytokines would further our understanding of the regulatory mechanisms in tissue fibrosis. Here, we report the effect of multiple cytokine combinations on primary HSCs. We first examined the effect of individual cytokines and then the simultaneous exposure of different cytokines, including interleukin-6 (IL-6), IL-1 α (IL-1 α), platelet-derived

growth factor (PDGF), tumour necrosis factor- α (TNF- α) and transforming growth factor- β (TGF- β), on matrix metalloproteinase-1 (MMP1) gene expression in primary HSCs. We observed that the combination of all five cytokines induced higher levels of MMP1 gene expression. Of these cytokines, TNF- α and IL-1 α were found to be the key cytokines for not only inducing MMP1 expression, but also increasing α -smooth muscle actin gene expression. In conclusion, the combined treatment of TNF- α and IL-1 α on HSCs had an enhanced effect on the expression of the fibrotic genes, MMP1 and α -smooth muscle actin, so appears to be an important regulator for tissue regeneration. This finding suggests that stimulation with combined anti-fibrotic cytokines is a potential approach in the development of a novel therapy for the recovery of liver fibrosis.

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Introduction

Fibrosis is a common pathological feature of chronic inflammation. In response to injury, activated fibroblasts accumulate in the damaged tissue and irreversibly deposit excessive amounts of extracellular matrix (ECM) components, which severely impair organ function (Coker and Laurent 1998; Gabay and McInnes 2009; Borthwick et al. 2013). In the liver, the

most common disease is liver fibrosis, which is induced by a number of factors including liver inflammation during obesity, viral infection and alcohol overdose, to name a few (Poynard et al. 1997; Angulo et al. 1999). Within fibrotic lesions, fibroblastic cells are activated by cytokines and growth factors such as TGF- β , and are often found to express alpha-smooth muscle actin (α -SMA) and adopt a myofibroblast phenotype with autocrine secretion (Gressner and Weiskirchen 2006; Seki et al. 2007). As the major producers of ECM components, including collagen type I, myofibroblasts play a central role in the pathogenesis of fibrosis. During the process of liver regeneration, the ECM must be degraded by proteases (Lee et al. 1995; Fausto et al. 2012). For this to occur, hepatic stellate cells (HSCs) become the central organisers for remodelling of the liver by producing MMPs to breakdown the ECM. MMPs are also involved in normal physiological processes, such as tissue remodelling, as well as in disease processes, such as arthritis and metastasis (Kleiner and Stetler-Stevenson 1999; McInnes and Schett 2007). More specifically, MMP-1 breaks down interstitial collagen type-I, -II and -III (Brenner et al. 2012; Mak et al. 2012). However, in the late stage of liver fibrosis, HSCs produce tissue inhibitor of metalloproteinase (TIMP) to inhibit the MMPs themselves. These MMPs and TIMPs are regulated by inflammatory cytokines, such as TNF- α , TGF- β , IL-1 α and IL-6. In the hepatitis condition, non-parenchymal cells produce various cytokines that influence HSCs simultaneously (Benyon and Iredale 2000; Hemmann et al. 2007). The majority of previous investigations report the effects of single cytokines on HSCs (Tarrats et al. 2011; Han et al. 2004). However, investigating the effects of combined cytokines is necessary to better understand the pathogenesis of liver fibrosis. Here, we describe the effect of multiple cytokines on primary HSCs and the LX-2 cell line.

Materials and methods

Cell culture

Primary HSCs (Nakamura et al. 2010) and LX-2 cells (Xu et al. 2005) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nippon Suisan Kaisha, Ltd., Tokyo, Japan) supplemented with 20 and 2 %

foetal bovine serum (Biowest, Inc., Nuaillé, France), respectively, with 100 U/mL penicillin and 100 μ g/mL streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C in a humidified atmosphere of 5 % CO₂. When cytokine stimulation was carried out, primary HSCs and LX-2 cells were exposed to cytokine concentrations of 1 ng/mL for each following serum starvation for 24 h using DMEM/0.5 % serum. Recombinant human TNF- α , IL-1 α , IL-6 and PDGF were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human TGF- β was purchased from Peprotech Inc. (Rocky Hill, NJ, USA).

RNA extraction, reverse transcription and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the cells using Trizol reagent (Life Technologies, Carlsbad, CA, USA). Reverse-transcribed complementary DNA was synthesized from 200 ng total RNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, South San Francisco, CA, USA) and was subsequently used for real-time qPCR using the KAPA SYBR FAST qPCR Universal Kit (Kapa Biosystems, Wilmington, MA, USA). The primer sequences (5'-3') used for PCR were as follows: glyceraldehyde-3-phosphate dehydrogenase (forward, 5'-CTGGCTACACTGAGCACCA-3' and reverse, 5'-ACATGACAAGGTGCGGCTCC-3'); MMP1 (forward, 5'-ACTCTGGAGTAATGTACACCT-3' and reverse, 5'-GTTGGTCCACCTTTTCATCTTCA-3'); α -SMA (forward, 5'-CAGGGCTGTTTTCCCATC CAT-3' and reverse, 5'-ACGTAGCTGTCTTTTGT TCCC-3'). The relative gene expression of MMP1 and α -SMA was calculated using the 2- $\Delta\Delta$ Ct method. Data are presented as the relative quantity of target mRNA normalised to the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Cell culture and cytokine assay

LX-2 cells and primary HSCs were cultured in DMEM supplemented with 2 and 20 % foetal bovine serum with 100 U/mL penicillin and 100 μ g/mL streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO₂ overnight. When cytokine stimulation was carried out, primary HSCs

and LX-2 cells were exposed to cytokine concentrations of 1 ng/mL for each following serum starvation for 24 h using DMEM/0.5 % serum.

Immunocytochemistry

For immunofluorescence labelling, cells were fixed in 4 % paraformaldehyde, and incubated with a primary antibody for 1 h at 4 °C (Nakamura et al. 2010). The primary antibody was monoclonal mouse anti-human SMA (Clone 1A4) (Agilent Technologies, Inc., Santa Clara, CA, USA), with a secondary Cy3-conjugated AffiniPure anti-mouse IgG (Jackson ImmunoResearch, Inc., West Grove, PA, USA). After mounting, the slides were viewed using a confocal microscope (Olympus America, Center Valley, PA, USA).

Results

Effect of inflammatory cytokines on primary HSCs

Following liver damage by injury, virus or oxidative stress, Kupffer cells secrete multiple inflammatory-related cytokines, such as IL-6, IL-1 α , TNF- α and TGF- β (Chensue et al. 1991; Thurman 1998; Kolios et al. 2006), which regulate ECM production by HSCs for liver regeneration. To investigate the effect of these inflammatory cytokines on HSCs, we prepared primary human HSCs from excised human livers (Nakamura et al. 2010) prior to assaying.

First, we investigated the effect of these inflammatory cytokines on MMP1 expression in primary HSCs. As shown in Fig. 1, only IL-1 α induced MMP1 expression by threefold in primary HSCs, whilst IL-6, PDGF, TNF- α and TGF- β had no effect. This effect was observed using primary HSCs obtained from the liver of two different patients, indicating a generic response between patients. MMP1 gene expression is reported to be induced by TNF- α in many types of fibroblasts (Sasaki et al. 2000; Xu et al. 2011). However, in our primary HSCs, TNF- α barely induced MMP1 expression. We then examined the effect of combined inflammatory cytokines and observed that simultaneous exposure of primary HSCs to IL-6, IL-1 α , PDGF, TNF- α and TGF- β dramatically induced MMP1 expression. The MMP-1 induction level was 5- to 10-fold higher than the individual effect of IL-1 α . This indicated that a combination of inflammatory

cytokine signals enhanced IL-1 α to further induce MMP1 expression.

To determine which of the cytokines enhanced the IL-1 α signal, we examined the cytokine effect after withdrawal of individual cytokines from the five-cytokine mixture on primary HSCs. Figure 2 shows that both IL-1 α and TNF- α were necessary for increased MMP1 expression in primary HSCs; therefore, TNF- α exposure likely enhanced the IL-1 α signal.

Effect of IL-1 α and TNF- α on MMP1 expression in primary HSCs

We examined the effect of combined IL-1 α and TNF- α on MMP1 expression in primary HSCs. TNF- α and IL-1 α increased MMP1 expression 2- and 5-fold higher than the control, respectively (Fig. 3). Moreover, the simultaneous addition of IL-1 α and TNF- α induced MMP1 gene expression synergistically in primary HSCs. Although the MMP1 increased levels differed among patients (Fig. 3, panel A and B), the synergistic effect of the combined cytokines was similar among patients (Fig. 1). This indicated that IL-1 α and TNF- α are key regulators for MMP1 expression in primary HSCs.

Effect of IL-1 α and TNF- α on α -SMA expression in primary HSCs

A number of studies have previously reported that α -SMA gene expression is high in activated HSCs, causing tissues to enter a fibrotic state (Brenner et al. 2012; Rockey et al. 2013). Here, we examined the effect of cytokines on α -SMA gene expression in primary HSCs. Interestingly, TNF- α and IL-1 α decreased α -SMA gene expression by half and a quarter, respectively (Fig. 4). Furthermore, simultaneous exposure with TNF- α and IL-1 α reduced the α -SMA level by a fifth (Fig. 4). These results suggest that both TNF- α and IL-1 α regulate the expression of fibrotic genes in addition to MMP1 gene expression, which is a controlling gene for the fibrosis protein.

Effect of IL-1 α and TNF- α on α -SMA protein in primary HSCs

As shown above, IL-1 α and TNF- α were capable of drastically changing the MMP1 and α -SMA

Fig. 1 Effect of inflammatory cytokines on MMP1 expression in primary HSCs. Primary HSCs were isolated from excised human livers. Following serum starvation, TGF-β, IL-1α, IL-6, TNF-α and PDGF were added to the medium at 1.0 ng/mL and the cells were incubated for a further 24 h. MMP1 mRNA expression was measured by qPCR. Untreated cells were used as

a control. The results derived from two patients are shown (panel **A** and **B**). Because only a low number of HSCs can be obtained from a hepatocellular carcinoma patient liver, experiments using identical HSCs could not be repeated. However, the synergistic effect of the combined cytokines was similar among two different patients

Fig. 2 Identification of cytokines enhancing MMP1 expression in primary HSCs. Primary HSCs were isolated from excised human livers. Following serum starvation, TGF-β, IL-1α, IL-6, TNF-α and PDGF were added to the medium at 1.0 ng/mL as

indicated in Figure and the cells were incubated for a further 24 h. MMP1 mRNA expression was measured by qPCR. Untreated cells were used as a control. The results derived from two patients are shown (panel **A** and **B**)

expression levels. However, cell morphology was unchanged during cytokine stimulation. We therefore examined the amount and distribution of α-SMA protein in primary HSCs using immunohistochemistry.

In the untreated cells, a high amount of α-SMA protein was detected throughout the cell cytoplasm (Fig. 5). Following TNF-α stimulation, although there was no change in the amount and distribution of α-SMA

Fig. 3 Effect of TNF- α and IL-1 α on MMP1 expression in primary HSCs. TNF- α and IL-1 α cytokines were simultaneously exposed to primary HSCs at 1.0 ng/mL for 24 h. MMP1 mRNA expression levels were measured after exposure to individual

and combined cytokines using qPCR. Untreated HSCs were used as a control. The results derived from two patients are shown (panel A and B). Each bar represents the means and standard deviations of three independent experiments

Fig. 4 Effect of TNF- α and IL-1 α on α -SMA expression in primary HSCs. TNF- α and IL-1 α cytokines were simultaneously exposed to primary HSCs at 1.0 ng/mL for 24 h. α -SMA mRNA expression levels were measured after exposure to individual

and combined cytokines using qPCR. Untreated HSCs were used as a control. The results derived from two patients are shown (panel A and B). Each bar represents the means and standard deviations of three independent experiments

protein in the primary HSCs, IL-1 α stimulation slightly reduced the amount of α -SMA protein. Furthermore, the simultaneous exposure of both IL-1 α and TNF- α dramatically decreased the protein level and also the distribution of α -SMA protein into filamentous shapes in the primary HSCs. These observations indicate that the signals obtained from both IL-1 α and TNF- α may contribute to repair of the fibrotic state through various mechanisms in HSCs.

Effect of IL-1 α and TNF- α on MMP1 and α -SMA expression in LX-2 cells

Investigation using primary HSCs and combined inflammatory cytokines suggested that IL-1 α and TNF- α have regulatory roles in MMP1 and α -SMA

expression. We next confirmed their effect on MMP1 expression using the HSC cell line, LX-2, which was generated by SV40 T antigen spontaneous immortalisation in low-serum conditions (Xu et al. 2005).

We examined MMP1 and α -SMA gene expression in LX-2 cells following IL-1 α and TNF- α stimulation. When used individually, TNF- α and IL-1 α increased MMP1 expression by approximately 2- and 5-fold in LX-2 cells, respectively, whilst the simultaneous stimulation with TNF- α and IL-1 α induced MMP1 expression by approximately sevenfold (Fig. 6A). With respect to α -SMA gene expression, a reciprocal effect to MMP1 gene expression was observed (Fig. 6B). This indicates that the response of both primary HSCs and the LX-2 cell line to inflammatory cytokines was comparable; therefore, LX-2 cells could

Fig. 5 Attenuation of α -SMA protein expression in primary HSC by TNF- α and IL-1 α . Following serum starvation, IL-1 α and TNF- α were added to the medium at 1.0 ng/mL and

incubated for further 24 h. The cells were fixed and α -SMA protein was identified by immunohistochemistry

Fig. 6 Effect of TNF- α and IL-1 α on MMP1 and α -SMA expression in the LX2 cell line. Following serum starvation, IL-1 α and TNF- α were added to the LX2 cultured medium at 1.0 ng/mL and cells were incubated for a further 24 h. MMP1

and α -SMA mRNA expression was measured by qPCR. Untreated cells were used as a control. Each *bar* represents the means and standard deviations of five independent experiments

be used in subsequent experimentation to identify mechanisms for the regulation of fibrotic genes, such as MMP1 and α -SMA, and to develop a therapy to cure liver fibrosis.

Discussion

Liver fibrosis is characterised by excessive accumulation of ECM proteins, including collagen, which occurs in most types of chronic liver disease (Bataller and Brenner 2005; Friedman 2008; Wells 2005).

Advanced liver fibrosis results in liver failure and portal hypertension, and often requires liver transplantation. Therefore, the development of an efficient therapy for liver fibrosis is urgently needed. MMPs actively degrade collagens and are reported to be regulated by TNF- α in rheumatoid arthritis (Choy and Panayi 2001; Firestein et al. 1990). Here, we have shown that TNF- α itself elicited only a weak effect on MMP1 expression, but when combined with IL-1 α , MMP1 expression was dramatically upregulated in both primary HSCs and the established LX-2 cell line. Additionally, TNF- α and IL-1 α had a reciprocal effect

on α -SMA expression compared with MMP1 expression. These results suggest treatment with a combination of cytokines, namely IL-1 α and TNF- α , could be effective in regulating the fibrotic genes in HSCs and therefore in the fibrotic liver.

To develop a therapy to cure liver fibrosis, it will be important to consider how different cytokine signals cross-talk to regulate fibrotic gene expression. Both IL-1 α and TNF- α cell signalling occur via mitogen-activated protein kinase (MAPK) signalling pathways positioned downstream of the IL-1R1 and TNF receptor families (Foey et al. 1998; Mengshol et al. 2001; Tran et al. 2001). Within MAPK signalling there are three main pathways: p38; extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase1/2 (Seger and Krebs 1995; Johnson and Lapadat 2002). These pathways are known to directly or indirectly interact with nuclear factors to modify transcription of their target gene expression. It is likely that the cross-talk point for the combined effect of inflammatory cytokines exists on or near to the MAPK signalling pathways.

As a preliminary investigation, we have observed the roles of p38 and ERK for the regulation through IL-1R1 and TNF receptor in knockdown experiments (results not shown, manuscript in preparation). In addition, previous studies have identified the promoter region of MMP1, including the binding site of the transcription factors activator protein-1 (AP-1) and CRE-binding protein (Chambers et al. 2013; Reunanen et al. 2002). Among them, AP-1 is known to be regulated by TNF- α and IL-1 via nuclear factor-kappa B and Nuclear factor NF-kappa-B inhibitor kinase signalling in several fibroblasts (Cheng et al. 2009; Xu et al. 2011). Therefore, AP-1 seems to be a candidate transcription factor for the regulation of fibrotic gene expression.

In conclusion, our observations reveal that combined inflammatory cytokines dramatically induced MMP1 gene expression in both human primary HSCs and the LX-2 cell line. In particular, TNF- α and IL-1 α were the most effective cytokine combination. Reciprocally, α -SMA gene expression was reduced following stimulation with TNF- α and IL-1 α . These results indicate that the inflammatory cytokines, TNF- α and IL-1 α , act as anti-fibrotic factors on human primary HSCs. In addition, the cross-talk point of this synergistic phenomenon following simultaneous cytokine IL-1 α and TNF- α exposure remains to be elucidated.

Identifying the key factor for this synergistic phenomenon could provide a novel therapeutic approach to treating liver fibrosis.

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