Expression of CXC chemokine IP-10/Mob-1 by primary hepatocytes following heat shock

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ABSTRACT

Objective: To examine the expression of chemokine IP-10/Mob-1 of hepatocytes in responses to the stress imposed during isolation by collagenase perfusion.

Methods: This study was performed in the Faculty of Life Sciences University of Manchester during 2001-2005. We employed western and northern blotting analysis to detect IP-10/Mob-1 in isolated and cultured hepatocytes in response to isolation stresses and under heat shock stimulation in this project.

Results: We showed that the ELR$^-$CXC chemokine, IP-10/Mob-1 is secreted from isolated rat hepatocytes immediately after isolation and early during culture and IP-10/Mob-1, expression by hepatocytes was also stimulated in response to heat shock.

Conclusion: It seems that hepatocytes mimic the experiences of liver injury in vivo such as during stress, trauma, or after insults, and therefore, produce stress related agents like IP-10/Mob-1 chemokine to overcome such a injurious condition following isolation and heat shock stimulation. This study also provides a useful model to study the regulation of expression of this chemokines *in vitro.*

Saudi Med J 2007; Vol. 28 (4): 514-518

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Received 24th June 2006. Accepted 27th November 2006.

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Liver is one of the most important organs of the body and is involved in several aspects of metabolism.[1] Within the liver cells, parenchymal cells undertake the majority of liver-specific functions.[1,2] Hence, isolated and *in vitro* cultured hepatocytes are used globally in both industry and academia.[3] Several known injurious conditions such as hepatitis, ischaemia/reperfusion, sepsis/endotoxemia, and drugs causes recruitment of macrophages, neutrophils, and other immune cells to the liver.[4] Neutrophils are recruited to the hepatic vasculature following local liver injury, and consequent CXC chemokine production or exposure of liver to inflammatory cytokines.[5,6] Involvement of CC chemokines such as macrophage chemotactic factor-1 (MCP-1), ELR$^+$ CXC chemokines (such as IL-8/MIP-2), and ELR$^-$ chemokines (such as IP-10/Mob-1) in acetaminophen-induced liver injuries, and modulation of liver regeneration processes, has been reported.[7-10] Enhanced expression of interferon gamma inducible protein-10 (IP-10)/Mob-1 has also been detected in liver in conditions of abnormalities. There is no detectable IP-10/Mob-1 in normal liver *in vivo*, however, higher expression of IP-10 has been shown to occur in response to hepatic injury and partial hepatectomy, alcoholic liver disease and liver sepsis.[11-15] Several independent research groups have documented the stimulation of expression of IP-10/Mob-1 subsequent to hepatocyte isolation.[16,17] Given these brief introductory facts, we assumed that during the processes of liver cell isolation by collagenase perfusion and early culture, hepatocytes experience a stressful condition similar to the situations they encounter in cases of liver injuries (such as, insults, trauma, infection, and inflammation including some diseases states). Thus, in the process of isolation and culture, some stress-related signals will be activated, leading hepatocytes to enter a response homologous to the stress response that occurs in immune responses, characterized with early expression of some mediators including chemokines to overcome the injurious situation. Therefore, this work aimed to examine early signaling events that are triggered, and activated by hepatocyte isolation and early culture, and how they influence hepatocytes response to these types of conditions. Hence, we have chosen one of

the stress mediators (CXC chemokine IP-10/Mob-1) for analysis.

Methods. ***Perfusion, isolation, and maintenance of hepatocytes in culture.*** Hepatocytes were obtained from fed male Sprague-Dawley rats (BSU, University of Manchester) weighing approximately 200 g. Hepatocytes were isolated from rats by perfusion of the liver with Krebs-Henseleit bicarbonate (128 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$)/collagenase (Sigma, Poole, UK) under aseptic conditions. After 10 minutes, the liver was removed and under sterile conditions, the liver was gently broken down and filtered through sterile gauze with Krebs-Henseleit bicarbonate. The cells were washed 3 times by differential centrifugation to harvest a population of large parenchymal cells and each time gently resuspending the pellet in Krebs-Henseleit bicarbonate. The final pellet was resuspended in inoculation medium (serum-free Waymouths MB/721 media; Invitrogen Ltd, Paisley, Scotland, UK) and the viability of the cells was assessed using trypan blue. The hepatocytes were used only if they were >85% viable and were generally 90-95% viable. The hepatocytes were of higher purity, and under the light microscope, endothelial cells were rare, (never more than 1% of the population). Random batches of cells were checked for endothelial cell contamination using specific antibody immunofluorescence with antibodies to von Willebrands factor (Santa Cruz Biotechnology, California, USA). The hepatocytes were seeded (2×10^6 cell/ml) on to collagen type 1-coated plates (3-cm plates for ribonucleic acid (RNA) and 6-cm plates for protein) and cultured in inoculation medium at 37°C under an atmosphere of 5% CO_2 in O_2. After 3 hours in culture, the media on the cells was replaced with maintenance medium (Waymouths MB/721 media supplemented with bovine serum albumin (BSA) [0.2% w/v] and sodium oleate [0.0005% w/v]). The cells were treated as described in the figure legends.

Cloning. The amplified fragment of complementary DNA (cDNA) (approximately 50 ng) was ligated into pUAg cloning vector (100 ng) (Ingenius, R & D Systems Europe Ltd, Abingdon, UK) by incubation with T4 ligase (1 Weiss unit) and ligase reaction buffer (30 mM Tris–HCl, pH 7.8 containing 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP) to a final volume of 10 µL for 16 hours at 4°C. The vector was transformed into *Escherichia coli* XL-1 Blue and grown on Luria-Broth (LB) plates containing ampicillin (50 µg/ml) overnight at 37°C. Colonies were selected and cultured overnight at 37°C in LB media containing ampicillin as before. From this overnight culture, the pUAg vector with the ligated cDNA fragment was isolated using the Plasmid Midi kit (Qiagen Ltd, Dorking, Surrey, UK). The cDNA fragment was excised from the pUAg vector using the restriction enzyme Hind III (Boehringer Mannheim, Sussex, UK) for 1 hour at 37°C. The restriction digest was electrophoresed on a 1% agarose gel at 70 V for 1 hour. The cDNA fragment was excised from the gel and purified from the agarose using the Gel Extraction Kit (Qiagen) and sequenced using dye-terminator chemistry.

Northern blot analysis. The RNA (20 µg) was electrophoresed on a 1% agarose/17% formaldehyde gel before being transferred and fixed onto Hybond-N™ nitrocellulose membrane (Amersham International, Aylesbury, UK). Part-length cDNA of IP-10/Mob-1 was random labeled with (α-^{32}P) dATP (50 µCi) (ICN Biomedicals Ltd). To standardize the RNA loading, the filters were reprobed with 18S rRNA, which was labeled with [α-^{32}P] dATP (20 µCi) using Nick translation. The membranes were prehybridized at 42°C in 50% (v/v) formamide containing 5× sodium-salt-phosphoric acid-EDTA 2× Denhardt's, 0.1% (w/v) sodium dodecyl sulphate (SDS), and 0.1 mg/ml heat-denatured salmon sperm DNA. The membranes were then hybridized with one of the labeled cDNA oligonucleotide probes in fresh hybridization mixture at 42°C overnight. The membranes were washed twice for 15 min at room temperature with 2× SSC/0.1% (w/v) SDS, before a final 20 minutes wash at 55°C with 0.1× SSC/0.1% (w/v) SDS. Then the membranes were autoradiographed using intensifying screens at -70°C. Quantitation of the hybridization intensity was by phosphorimage analysis.

Western blot analysis. At indicated time points, medium was removed from hepatocyte cultures and centrifuged. Clarified supernatants from 0-hour and incubated samples were used for Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting and densitometry was performed to quantify the expression of IP-10/Mob-1. Equal amounts of protein (35 g) were loaded and resolved on a 10% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking with 3% (w/v) milk in phosphate-buffered saline (PBS)/Tween (10 mM Tris, pH 7.4 containing 140 mM NaCl, 0.1% [v/v] Tween 20), the nitrocellulose membrane was incubated overnight at 4°C in PBS/Tween containing 3% (w/v) milk including anti-rat IP-10 (Chemokine.com, Houston, USA supplied by AMS Biotechnology, Abingdon, UK). Subsequently, anti-rabbit horseradish peroxidase-conjugated antibodies (diluted, 1:1000) were used accordingly, and the enhanced chemiluminescence (ECL) detection system (Amersham International) were used to define protein localization and amount.

Statistical analysis. All data are expressed as mean ± SEM. Comparisons of variables between 2 groups

were performed using an unpaired Student's *t* test. Differences were considered significant when $P<0.05$.

Results. To examine the specific chemokines produced specific CXC chemokine IP-10/Mob-1, was selected for detailed examination, and western and northern blotting methods were applied to assess the expression of this CXC chemokine.

A) Expression of CXC chemokines at protein and messenger RNA (mRNA) level during basal culture of hepatocytes. As shown in **Figure 1,** IP-10/Mob-1 protein was weakly detectable in freshly isolated hepatocytes (0-hour), and the expression of IP-10/Mob-1 increased dramatically as a result of hepatocyte isolation, but decreased after 27 hours of culture. Statistical analysis showed that there is a significant difference between the expression of IP-10/Mob-1 at 1 hour compared to all other time points of culture (**Figure 1).** IP-10/Mob-1, cDNA species were used as probes in northern blotting analysis. As is obvious from **Figure 2,** IP-10/Mob-1 mRNA was not expressed in freshly isolated liver cells (0-hour), but was increased significantly at 3 hours before decreasing with subsequent culture.

B) Analysis of expression of IP-10/Mob-1 protein in response to heat shock. As is clear from **Figure 3,** heat shock produced significant stimulation in expression of IP-10/Mob-1. Although the precise pattern varied slightly with each chemokine, it was clear that profound stimulation was observed over a subsequent period of 24 hours. The maximum activation of expression was exhibited within 3 hours of heat treatment. There were relatively small decreases in expression as culture progressed (up to 24 hours).

Discussion. Isolation of hepatocytes is an invasive process that alters cell-matrix and cell-cell interactions within the liver.[16] Hence, in part, cell isolation mimics injury to the liver. Comparison of isolation conditions and liver disorders may aid in identification of factors involved in the response of liver to the changes in the environment during hepatocyte isolation. In agreement with these results several research groups showed that isolated and cultured hepatocytes and

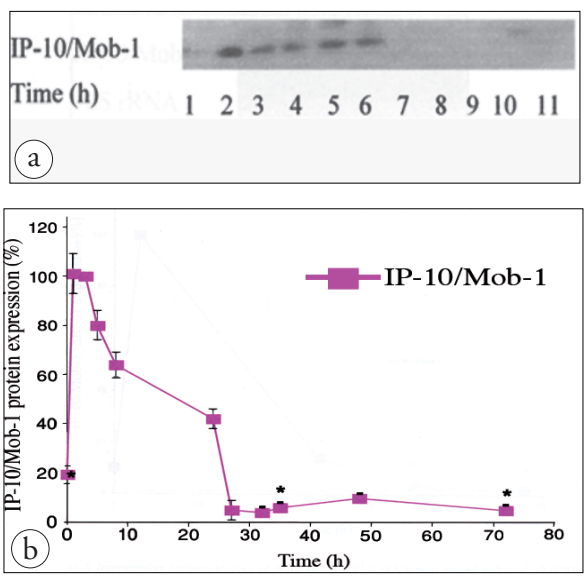

Figure 1 - Expression of IP-10/Mob-1 at protein level by hepatocytes during primary culture. **a)** Representative profile of protein bands from western blotting of IP-10/Mob-1 expression following hepatocytes isolation and early culture. lane 1= 0-hour (freshly isolated hepatocytes); lane 2= 1 hour; lane 3= 3 hours; lane 4= 5 hours; lane 5= 8 hours; lane 6= 24 hours; lane 7= 27 hours; lane 8= 32 hours; lane 9= 35 hours; lane 10= 48 hours and lane 11= 72 hours. **b)** Time-dependent variation of IP-10/Mob-1 protein expression following hepatocytes isolation and early culture. The expression of IP-10/Mob-1 protein is sharply increased immediately after isolation and then significantly decreased with time. [**P*<0.05 v 100% value].

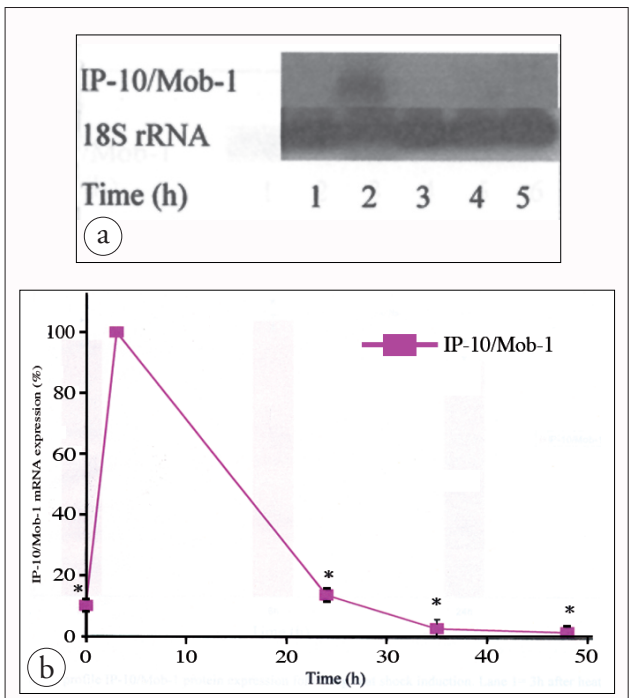

Figure 2 - Expression of IP-10/Mob-1 at mRNA level by hepatocytes during primary culture. **a)** A representative northern analysis of IP-10/Mob-1 mRNA/rRNA ratio probed with [α-^{32}p] dCTP - labeled IP-10/Mob-1, cDNA. 18S rRNA was used to indicate for loading equivalence. lane 1= 0-hour; lane 2= 3 hours; lane 3= 24 hours; lane 4= 35 hours; lane 5= 48 hours. **b)** Time-dependent variation in IP-10/Mob-1 mRNA expression following hepatocytes isolation and early culture. The expression of IP-10/Mob-1 mRNA is sharply increased following hepatocytes isolation and decreased with time. [**P*<0.01 v 100% value].

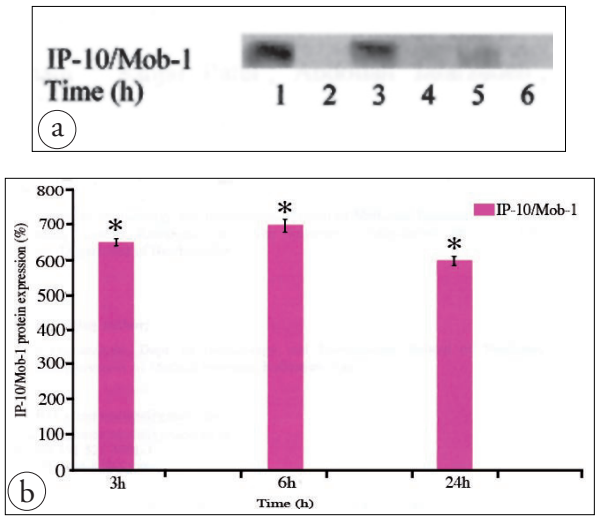

Figure 3 - Expression of IP-10/Mob-1, at protein level in response to heat shock. **a)** Representative profile IP-10/Mob-1 protein expression following heat shock induction. Lane 1= 3 hours after heat shock treatment; lane 2= 3 hours control; lane 3= 6 hours after heat shock treatment; lane 4= 6 hours control; lane 5= 24 hours after heat shock treatment; lane 6= 24 hours control. **b)** Time-dependent variation in expression of IP-10/Mob-1 protein following heat shock stress on hepatocytes culture. The relevant control at each time point was assigned as 100% and the expression of chemokines in response to heat shock was compared with the relevant control. [**P*<0.05 v relevant time control].

hepatoma cells express several different chemokines.[16-18] Although, induction of chemokine gene expression in macrophages, fibroblasts, and osteoblasts is regulated by both transcriptional and post-transcriptional and cell type-dependent pathways,[19] the mechanisms that regulate chemokine gene expression in hepatocytes is yet to be fully identified. However, the induction of IP-10/Mob-1 mRNA expression within only few minutes to a few hours of hepatocyte isolation suggests that the regulation is likely to be at transcriptional level. It was notable that the amount of IP-10/Mob-1 decreased in the absence of medium changes (such as in the period between 3 hours and 24 hours of culture). This could be due to binding of these chemokines to their specific receptor and internalization or due to proteolysis by an unknown protease secreted by hepatocytes into the medium.

The promoters for IP-10/Mob-1[19] has been shown to have consensus response elements for binding of nuclear factor kappa B (NF-κB), and the NF-κB pathway may allow for a mechanism to activate expression of these 3 chemokines specifically. It is worth stressing that there was an evidence for decreased of IP-10/Mob-1 at protein level as culture progressed without medium changes **(Figure 1).** The expression of CXC chemokines after hepatocyte isolation could be due to perfusion, isolation, and the initial wash stages of the isolation procedure. However, due to the absence of detectable amounts of IP-10/Mob-1, protein *in vivo*,[8,17,20,21] one possible mechanism of increased expression of these IP-10/Mob-1 *in vitro* during perfusion is from physiological changes for the hepatocyte environment during the isolation procedures, which could arise from damage to the cells, or removal of cells from cell-cell and cell-matrix interactions. Signalling events triggered by other external stresses, such as heat shock, oxidative, and osmolarity stresses, regulate intermediates that are common to events triggered by pro-inflammatory cytokines.[22,23] Heat shock activates signalling pathways with very notable changes in c-jun N-terminal kinase (JNK) and p38 isoforms and their upstream activators such as Mitogen-activated-protein-kinase-kinase-3 (MKK3) and Mitogen-activated-protein-kinase-kinase-6 (MKK6.)[22-24] In the present study, we have shown that in response to heat shock, all of the studied IP-10/Mob-1 exhibited was increased the expression. In response to heat shock, a variety of other genes including, heat shock proteins (HSPs) (especially HSP27) are known to be activated by a protein kinase downstream of p38.[25] Thus, the expression of various HSPs has been shown to occur in the liver in response to heat shock. HSP70 expression also increased in hepatectomy and has been observed in proliferating hepatocytes, and HSP25 expression was elevated in liver carcinomas.[19] A heat shock response element has been demonstrated in the promoter of IP-10/Mob-1 by Ohmori and Hamilton,[24] and the induction of expression of this gene could be explained as a part of this element. The heat shock stimulation may modulate some other unknown regulatory elements in those genes or produce secondary effects through other regulatory genes (potentially from pro-inflammatory cytokines to growth factors) and indirectly induce expression chemokines. Hence, early signalling events that are activated during hepatocyte isolation and early culture provides an initial point for future studies to the development of more reliable models for the study of the liver function. Therefore, one of the main aspects of future work then would be identification of the initiators of these signalling responses in hepatocytes and liver injuries. This would be a valuable model for understanding regulation of liver function and differentiation in relation to liver injuries and hepatocyte isolation.

Acknowledgments. *We would like to thank all the members of Lab 2.300 (both past and present), for their help and patience, and for making life in the lab a very enjoyable experience. A heartfelt thank also to the Ministry of Health and Medical Education of Islamic Republic of Iran for financial supports.*

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