Expression of Stromal Derived Factor Alpha (SDF-1α) by Primary Hepatocytes Following Isolation and Heat Shock Stimulation

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ABSTRACT

Stromal derived factor-α (SDF-1-α) is a CXC chemokine which has been demonstrated as a recruitment factor for leukocytes to the site of inflammation, infection, injury and following stress. This chemokine has been shown to be expressed by liver cells and in liver diseases. Hence, the aim of this study was to examine the expression of SDF-1 by hepatocytes in responses to the stress imposed during isolation by collagenase perfusion and under heat shock stimulation.

In this study hepatocytes (2-5 x 10⁶) were isolated from male Sprague Dawley rat liver and cultured in plates that were pre-coated with collagen Type-I matrix. The western and northern blotting analysis were employed to detect SDF-1 at protein and mRNA levels in isolated and cultured hepatocytes in response to isolation and heat shock stresses.

The SDF-1 is expressed by isolated rat hepatocytes immediately after isolation and early culture and decreased with time. SDF-1 protein was highly expressed in freshly isolated cells and decreased by time (27h) (P<0.05). mRNA was also expressed in freshly isolated cells (0h) but decreased after 24h of culture (P<0.01). This results also demonstrated that expression of SDF-1 by hepatocytes was increased in response to heat shock at different time points comparing with control (P<0.01).

These results demonstrated that the isolation and heat shock stresses induced the expression of SDF-1 in hepatocytes in a time-dependent manner. Accordingly, it seems that hepatocytes mimic the experiences that liver experience after injury in vivo and therefore, produce stress related agents like chemokines to overcome such a injurious condition.

Key words: Chemokine; Hepatocyte; Liver; SDF-1α

INTRODUCTION

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. Several known injurious conditions such as hepatitis, ischaemia/reperfusion, sepsis/endotoxemia and drugs cause recruitment of macrophages, neutrophils and other immune cells to the liver. Stomal derived factor-1 (SDF-1α and β) was initially identified in a bone marrow stromal cell line ST-2 and subsequently by expression cloning as a pre-B cell stimulating factor for stromal cells in the generation of B cells in vitro. High constitutive levels of SDF-1α have been observed in the non-inflamed biliary epithelium of the liver in association with CXCR4 expressing lymphocyte recruitment. Due to the lack of knowledge in analysis of the SDF-1α promoter, the exact mechanism of constitutive and ubiquitous expression of SDF-1α is not yet clear. Liver-infiltrating lymphocytes express CXCR4 receptor and it is thought that cells entering the non-inflamed liver may be attracted to, and retained at, the biliary epithelium, where they can provide immune surveillance against pathogens entering liver via the biliary tract. Immunohistochemical studies have revealed decreased level of SDF-1α protein in hepatocellular carcinoma, when compared with the other liver chronic diseases such as hepatitis C. SDF-1α is also produced by ductal plate cells (biliary epithelial cells progenitors) and it is involved in maturation and homing of B cells in fetal liver. We have already shown that the other chemokines of this subgroup such as IP-10 is expressed by hepatocytes during isolation, culture and following heat shock treatment and pro-inflammatory cytokines (TNF-α and IFN-γ) treatment. The expression of SDF-1 and Gro in H4 rat hepatoma cells under stimulation of different stimuli encouraged us to examine the expression of this chemokine in isolated and cultured primary hepatocytes to clarify possible mechanisms of chemokine expression by theses cell system. Thus it seems that, 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 to examine how they influence hepatocytes response to these types of conditions. Hence, we have chosen one of the stress mediators (CXC chemokine SDF-1) for analysis.

MATERIALS AND 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 KH2PO4, 1 mM MgSO4, 25 mM NaHCO3, 2.5 mM CaCl2)/collagenase (Sigma, Poole, UK) under aseptic conditions. After 10 min 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 three 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 greater than 85% viable and were generally 90–95% viable. The hepatocytes were of high 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 (2×10⁶ cell/ml) onto collagen type 1-coated plates (3-cm plates for RNA and 6-cm plates for protein) and cultured in inoculation medium at 37 °C under an atmosphere of 5% CO2 in O2. After 3 h in culture, the media on the cells was replaced with maintenance medium [Waymouths MB/721 media supplemented with 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 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 MgCl2, 10 mM DTT and 1 mM ATP) to a final volume of 10 μl for 16 h 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.
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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 h at 37 °C. The restriction digest was electrophoresed on a 1% agarose gel at 70 V for 1 h. 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**
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 SDF-1 was random labelled with [α-32P] dATP (50 μCi) (ICN Biomedicals Ltd). To standardize the RNA loading, the filters were reprobed with 18S rRNA which was labelled with [α-32P] dATP (20 μCi) using Nick translation. The membranes were prehybridized at 42 °C in 50% (v/v) formamide containing 5× SSPE, 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 labelled 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 min 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 0h and incubated samples were used for SDS-PAGE. Immunoblotting and densitometry were performed to quantify the expression of SDF-1. Equal amounts of protein (35 μg) were loaded and resolved on a 10% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. After blocking with 3% (w/v) milk in 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 in PBS/Tween containing 3% (w/v) milk including anti-rat SDF-1 (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 ECL detection system (Amersham International) were used to define protein localizaton and amount.

**Statistical Analysis**
All data are expressed as mean ± SEM. Comparisons of variables between two groups were performed using an unpaired Student's t test. Differences were considered significant when P<0.05.

**RESULTS**
The CXC chemokine SDF-1 was selected for detail examination and western and northern blotting methods were applied to assess the expression of this CXC chemokine.

The Cloning and Sequencing of SDF-1α Gene Fragment
The cloning and sequencing of SDF-1α gene fragment has been demonstrated in Figures 1 and 2.

**Figure 1. Restriction digest of the pCR®2.1 plasmid containing cDNA amplified using primers for SDF-1α.**
EcoRI digestion of the pCR®2.1 plasmid vector containing cDNA amplified using primers for SDF-1α. An Invitrogen TA cloning® kit was used for insertion of the PCR product into the plasmid vector. The vector was transformed into One Shot® cells (TOP10) and a mini-preparation performed. Fragments were resolved on a 1% (w/v) agarose gel. D= Digested and UD= Undigested. Numbers on the left denote the sizes of the bands for the molecular size marker.
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Sequence of rat SDF-1α (NMO22177) has been taken from the nucleotide database. The primers used for amplification are shown in the boxed region. Numbers denote successfully sequenced regions 1 and 2. 1* (sequenced region 1) shows identities between the sequence from the database and the cloned sequence (identities = 92/96; 95%). 2* (Sequenced region 2) showing identities between sequence from the database (shaded) and the cloned sequence (identities = 133/137; 97%). Red base denotes differences between sequencing data and database.

We have shown that the molecular size of SDF-1α gene fragment is approximately 1100 bp which is consisted with other previously observations. The sequencing of SDF-1α gene fragment is shown in Figure 2. The pattern of our sequenced fragment is exactly identical to the previously sequenced fragment exist in Pubmed nucleotide database with NMO22177 access number.

Figure 2. Sequencing data for SDF-1α

Figure 3. Expression of the CXC chemokine SDF-1 at protein level by hepatocytes during primary culture

A) Representative profile of protein bands from western blotting of SDF-1 expression following hepatocytes isolation and early culture. (lane 1 = 0h (freshly isolated hepatocytes); lane 2 = 1h; lane 3 = 3h; lane 4 = 5h; lane 5 = 8h; lane 6 = 24h; lane 7 = 27h; lane 8 = 32h; lane 9 = 35h; lane 10 = 48h and lane 11 = 72h

B) Time-dependent variation of SDF-1 protein expression following hepatocytes isolation and early culture. The expression of SDF-1 protein is sharply increased immediately after isolation and then significantly decreased with time. [*P< 0.05 v 100% value].

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A)

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

Expression of SDF-1α during Basal Culture of Hepatocytes

A. Protein level. As shown in Fig 3 the SDF-1α protein was highly expressed in freshly isolated hepatocytes (0h). In other words, the expression of SDF-1α protein sharply increased immediately after hepatocytes isolation. However, we have observed that the expression of SDF-1α protein significantly decreased with progression of the time up to 30 h after isolation and then after remains steady up to 80 h.

B. mRNA level. As shown in Fig 4 the expression of SDF-1α at mRNA level is parallel to the pattern of protein expression. The expression of mRNA markedly increased in fresh isolated hepatocytes with maximal expression that observed at 0 h. However, we have observed that the SDF-1α mRNA decreased with advanced times with sharply reduction at 24 h of culturing.

Analysis of expression of SDF-1 protein in response to heat shock

As is clear from Fig 5, heat shock had stimulated expression of SDF-1. As it is clear that profound stimulation was observed over a subsequent period of 6h. The maximum activation of expression was exhibited within 3h of heat treatment. There were relatively small decreases in expression as culture progressed (up to 24h).
DISCUSSION

Isolation of hepatocytes is an invasive process that alters cell-matrix and cell-cell interactions within the liver. Hence, in part, cell isolation mimics injury to the liver. In injury situations, such as liver regeneration, ischemia/reperfusion or in liver diseases (such as cancer and inflammation), defined signalling events are initiated and there are potential common events in relation to signalling initiation during liver injury and hepatocyte isolation. 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. Isolated and cultured hepatocytes express several different chemokines.\textsuperscript{15,16} Therefore, based on published data we chose to examine the expression of CXC chemokine SDF-1, known to be involved in many liver disorders. So there is clear evidence from our studies to indicate that SDF-1 is produced by isolated hepatocytes and that, in some cases, there is an activation of expression at mRNA level as a result of cell isolation and culture. SDF1-α expression occurred immediately after isolation of hepatocytes and continued for up to 24h as our group have shown that the other chemokines of CXC subgroup are expressed by hepatocytes during isolation, culture and following heat shock treatment and pro-inflammatory cytokine
We have shown that the expression of SDF-1α at both mRNA and protein levels markedly increased in freshly isolated hepatocytes. Although, due to technical problems we were unable to detect the in vivo expression of SDF-1α (before hepatocytes isolation), it seems the expression of both mRNA and protein can be attributed to isolation stresses. Before hepatocytes isolation the majority of cells are inactive and it seems that there is no adequately signals for highly expression of SDF-1α. In agreement with this chemokine in hepatocytes we also showed that the expression of SDF-1 and Gro in H4 rat hepatoma cells under stimulation of different stimuli including heat shock. Stimulation of mRNA expression and induction in response to hepatocyte isolation is likely to reflect differences at the level of promoter-regulatory elements (if this is due solely to transcriptional regulation). It would be of interest to carry out a comparative review of what is known of promoter regulatory elements for the SDF-1. The SDF-1α gene has been shown to contain binding motifs for the transcription factors SP1, CTF and to have CpG islands (a transcription binding motif specific for housekeeping genes). Therefore, the constitutive expression of SDF-1α appears to be due to these transcription factors, and the SDF-1α promoter lacks binding motifs for inducible factors such as NF-κB and AP-1. Heat shock activates changes in JNK and p38 isoforms and their upstream activators like MKK3 and MKK6. Signalling events triggered by heat shock, regulate intermediates that are common to events triggered by pro-inflammatory cytokines. In addition to isolation stresses in the present study, we have also shown that in response to heat shock SDF-1, exhibited increased 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. Thus the expression of various HSPs has been shown to occur in liver in response to heat shock. HSP70 expression also increased in hepatocyte and has been observed in proliferating hepatocytes and HSP25 expression was elevated in liver carcinomas. Specific heat shock response element has not been found in the promoter of SDF-1 but it may be modulated by some other unknown regulatory elements in this type of genes or produce secondary effects through other regulatory genes (perhaps like pro-inflammatory cytokines or growth factors) and indirectly induce expression SDF-1.

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