Expression of Gro (CXCL1) and SDF-1α (CXCL12) is differentially controlled by various intracellular signaling pathways in rat hepatocytes

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Chemokines play key roles in physiological and pathological activities of liver diseases. This study aimed to examine the intracellular signaling pathways involved in regulation of expression of Gro (CXCL1) and SDF-1α (CXCL12). Hepatocytes were isolated from rat liver and were cultured on collagen Type-I. Initially, cells were suspended in inoculation medium and then were added to the pre-wetted plates. Then the cells were treated with specified concentrations of inhibitor and incubated in 5% CO₂: 95% O₂ for 3 h. Medium was removed, centrifuged and separated using SDS-PAGE. The immunoblotting was used to analyze chemokines expression. The results demonstrated that at lower concentrations of SB203580, no detectable inhibition of Gro (CXCL1) and SDF-1α was observed, while there was a significant decrease in its expression at higher concentrations. Gro (CXCL1) was only decreased in the presence of the highest concentration of MG132 and the expression of SDF-1α (CXCL12) was not inhibited by MG132. KN62 inhibited expression of Gro (CXCL1) at higher concentrations but had no effect on SDF-1α (CXCL12) expression. Analysis of data showed that Stauorosporine produces a significant decrease in expression of it, but Gro (CXCL1) was only inhibited at 10 μM concentration of Stauorosporine. These data showed that SDF-1α (CXCL12) and Gro (CXCL1) are expressed following hepatocyte isolation and can be regulated by the inhibitors of p38, NF-KB, CAMK-II and stauorosporine. Furthermore, there may be further potential to prevent changes to hepatocyte phenotype and allow isolation of hepatocytes with a greater physiological phenotype.

Key words: Chemokine, hepatocyte, CXCL12, CXCL1.

INTRODUCTION

Chemokines play many key roles in physiological and pathological activities in infectious and inflammatory diseases, modulation of angiogenesis, tumor growth and stem cell proliferation (Luster, 1998). Chemokines are low molecular weight proteins (8-17 kDa). They are classified in four distinct groups as CXC, CC, CX3C and C. Depending on the presence or absence of a motif called ELR (Arg-Leu-Glu) before the first cysteine residue in their structure. CXC chemokines are also subdivided into ELR⁺ and ELR⁻. SDF-1α will fit in the category of ELR CXC chemokines (Baggiolini, 2001). Gro (CXCL1) is another that fit in the ELR⁺ CXC chemokines group. Expression of Gro (CXCL1) in hepatectomized mouse liver has been shown and it may play important role in hepatocyte proliferation and liver regeneration (Su et al., 2002). Increased Gro (CXCL1) expression is associated with some hepatic injuries and diseases including liver ischaemia/reperfusion in mouse and rat (Hisama et al., 1996), infection, paracetamol poisoning, sepsis and LPS.
injection in mice (Mercer-Jones et al., 1997; Salkowski et al., 1998). In human, elevated levels of Gro (CXCL1) have been shown in alcoholic hepatitis (Maltby et al., 1996). High constitutive levels of SDF-1α have been observed in the non-inflamed biliary epithelium of the liver (Coulomb-L’Hermin et al., 1999) in association with CXCR4 (SDF-1α receptor) expressing lymphocyte recruitment (Goddard et al., 2001; Terada et al., 2003).

SDF-1α is also produced by ductal plate cells (biliary epithelial cells progenitors), H4 rat hepatoma cells (Hassanshahi et al., 2006) and it is involved in maturation and homing of B cells in fetal liver (Coulomb-L’Hermin et al., 1999). 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 (Mitra et al., 1999). As other investigators have implicated the involvement of MAPK (Blinman et al., 2000; Ohmori and Hamilton, 1993) and NF-κB (Ren et al., 2002; Wang et al., 1999) (by using SB203580 and MG132, respectively) in regulation of IP-10 and JE by hepatocytes. The potential for changes in cellular Ca²⁺ (due to the use of EDTA and Ca²⁺ re-addition during perfusion) suggests a possible role for Ca²⁺ signalling. Many cellular responses to Ca²⁺ signals are also regulated by a group of multifunctional calcium-dependent calmodulin protein kinase such as CAMK-II and KN62 is an inhibitor of this pathway. Stauroporine possesses a wide variety of inhibitory effects on several protein kinases and serine/threonine kinases (Cho et al., 2003), cAMP-protein kinase, PKC (Johnson et al., 2002) and CAMK II (Liu et al., 2005). Observed changes in hepatocytes morphology and function in response to cell isolation are probably the result of initiation of cellular stresses. The signalling events related to stress such as MAPK (p38 and ERKs) and NF-κB, have previously been shown to be activated during hepatocyte isolation and early culture.

Given these brief introductory facts, we hypothesized 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.

**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 (Seglen, 1976). 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 incubation 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 to 95% viable. The hepatocytes were of high purity and, under the light microscope, endothelial cells were rare (never more than 1% of the population). The hepatocytes were seeded (2 × 10⁵ cell/ml) onto collagen type I-coated plates (3-cm plates for RNA and 6-cm plates for protein) and cultured in incubation 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.

**Western blot analysis**

After 3 h of culture, medium was removed from hepatocyte cultures and centrifuged and were used for SDS-PAGE. Immunoblotting and densitometry was performed to quantify the expression of Gro (CXCL1) and 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% (w/v) Tween 20), the nitrocellulose membrane was incubated overnight at 4°C 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 localization and amount.

**Statistical analysis**

All data are expressed as mean±SEM. Comparisons of variables between two groups were performed using an unpaired t-test. Differences were considered significant when P<0.05.

**RESULTS**

As it can be seen in Figure 1, in the presence of SB203580, the production of Gro (CXCL1) and SDF-1α was inhibited. Comparison of expression of SDF-1α in the presence and absence of different concentrations of SB203580 showed that significant decreases was observed at higher concentrations of the inhibitor. With Gro (CXCL1), a significant difference in expression was only observed in the presence of the highest concentration of inhibitor (50 μM) (Figure 1). As is obvious in Figure 2, there was no significant difference in expression of SDF-1α by primary hepatocytes over the entire range of MG132 concentration. With Gro (CXCL1), a significant difference in expression of Gro (CXCL1) was
Figure 1. Expression of Gro/KC and SDF-1α by hepatocytes in the presence and absence of SB203580. Representative protein bands from western blotting of Gro/KC and SDF-1α in the presence of different concentrations of SB203580 A). 1 = 1 µM of SB203580; 10 = 10 µM of SB203580; 20 = 20 µM of SB203580; 50 = 50 µM of SB203580; 0 (°C) = Control (no SB203580). Expression of Gro/KC and SDF-1α protein was quantified relative to that in basal culture of hepatocytes B). Expression of Gro/KC and SDF-1α protein in basal culture (control) was assigned as 100% and the expression of chemokines in the presence of different concentrations of SB203580 is related to this. Results are expressed as mean ± SEM for 4 independent experiments (*P< 0.05 v control).

only observed in presence of 50 µM of MG132 (Figure 2). KN62 inhibited expression of Gro (CXCL1) at highest concentrations but had no effect on SDF-1α expression (Figure 3). Expression of Gro (CXCL1) significantly decreased at 50 µM KN62. Analysis of data showed that Staurosporine produce a significant decrease in expression of SDF-1α with significant decrease in expression at 5 and 10 µM (Figure 4).

For Gro (CXCL1), the only significant difference in expression of Gro (CXCL1) was observed at 10 µM (Figure 4).

DISCUSSION

Following previous studies, in relation to expression of CXC chemokines by hepatocyte isolation and the effects of pro-inflammatory cytokines (Hassanshahi et al., 2007a;...
Figure 2. Expression of Gro/KC and SDF-1α by hepatocytes in the presence and absence of MG132. Representative protein bands from western blotting of Gro/KC and SDF-1α in the presence of different concentrations of SB203580 A). 1 = 1 µM of MG132; 10 = 10 µM of MG132; 20 = 20 µM of MG132; 50 = 50 µM of MG132; 0(°C) = Control (no MG132). Expression of Gro/KC and SDF-1α protein quantified relative to that in basal culture of hepatocytes B). Expression of Gro/KC and SDF-1α protein in basal culture (control) assigned as 100% and the expression of chemokines in the presence of different concentrations of MG132 is related to this. *Means significant difference in Gro/KC in the presence of 50 µM of MG132 compared with control (P < 0.05). Results are expressed as mean ± SEM for 4 independent experiments.
Figure 3. Expression of Gro/KC and SDF-1α by hepatocytes in the presence and absence of KN62. Representative protein bands from western blotting of SDF-1α in the presence of different concentrations of KN62 A). 1 = 1 µM of KN62; 10 = 10 µM of KN62; 20 = 20 µM of KN62; 50 = 50 µM of KN62; 0(°C) = Control (no KN62). Expression of Gro/KC and SDF-1α protein was quantified relative to that in basal culture of hepatocytes B). Expression of Gro/KC and SDF-1α protein in basal culture (control) was assigned as 100% and the expression of chemokines in the presence of different concentrations of KN62 is related to this. Results are expressed as mean ± SEM for 4 independent experiments (*P< 0.05 v control).

In particular, it is relevant that 20 µM SB203580 was used to inhibit the expression of Gro (CXCL1) and MCP-1 that occurred with isolation of acinar pancreatic cells (Blinman et al., 2000). Given the aforementioned information, the effects we have observed for SB203580 appear to occur within the acceptable concentration range reported by others and hence reflect a degree of sensitivity for inhibitory action.

The inability to totally inhibit Gro (CXCL1) and SDF-1α expression could be due to the involvement of other pathways in the control of expression of these chemokines. For example, at transcriptional level response elements for NF-κB are present in the promoters of these genes and SB203580 may not affect the NF-κB pathway (Ohmori and Hamilton, 1993; Wang et al., 1999). In another epithelial cell system (pancreatic cells), it has been demonstrated that expression of the chemokines Gro (CXCL1), MIP-2 and a CC chemokine...
MCP-1 requires p38 and that SB203580 blocked the stimulation of production of these chemokines in response to isolation of acinar pancreatic cells (Blinman et al., 2000). The Z-Leu-Leu-Leu-H (MG132) is a peptide aldehyde that is a substrate analogue of the proteasome and has been shown to be a proteosome inhibitor. Thus, in the presence of MG132, where proteosomal degradation of IκB is decreased, NF-κB will be retained in the cytoplasm. Inhibition of NF-κB:1κB complex dissociation has been shown to affect the expression of various genes that contain NF-κB consensus response elements in their promoter (Blinman et al., 2000). In this study, the expression of Gro (CXCL1) in early hepatocyte culture was inhibited by MG132 at concentrations greater than 20 μM. Gro (CXCL1) contains NF-κB consensus elements in its promoter (Table 1) and this appears to explain the inhibition of the Gro (CXCL1) expression in presence of MG132. In contrast to Gro (CXCL1), SDF-1α expression was not affected by inclusion of MG132.

The lack of response of this chemokine supports the importance of NF-κB consensus element in the regulatory network activated by hepatocyte isolation. In HepG2 cells, MG132 inhibited TNF-α and IL-1 induced IL-8 expression at a concentration of 25 μM and in acinar pancreatic cells, the expression of Gro (CXCL1) and MCP-1 that occurred due to cell isolation was inhibited by...
10 μM MG132 (Blinman et al., 2000; Joshi-Barve et al., 1993). By comparison to the concentration-dependency of results, it would appear that the effects of MG132 are operating at concentrations that reflect an action on proteosome function. Other studies in agreement with the results of this study in hepatocytes showed the involvement of NF-κB pathway in expression of Gro (CXCL1) and IP-10/Mob-1 in response to isolation (Ren et al., 2002; Wang et al., 1999). To date, there has been no other studies to show the effects of MG132 in chemokine expression by hepatocytes, but the inhibitory effects of MG132 have been shown for NF-κB and chemokine expression in acinar pancreatic cells (Blinman et al., 2000). In this study, Gro (CXCL1) was inhibited at the higher concentrations of KN62 (calmodulin and Ca²⁺/CAM K-Ⅱ pathway inhibitor) tested, while SDF-1 was insensitive to this inhibitor. Although, the exact mechanism of regulation of chemokine expression in relation to the inhibitory effects of KN62 is not clear, it probably involves a more common and general pathway of inhibition and may involve in reduction of other genes such as pro-inflammatory cytokines that may regulate the expression of these chemokines in hepatocytes.

Staurosporine (an alkaloid that arises from bacteria) has a wide variety of inhibitory and stimulatory effects. It has relatively non-specific inhibitory functions against a variety of Serine/Threonine protein kinases (Cho et al., 2003) including cAMP-dependent protein kinase and PKC (Johnson et al., 2002), cGMP-dependent protein kinase and Ca²⁺/CAMK-Ⅱ, MAPK (Johnson et al., 2002) and insulin receptor tyrosine kinase activity (Sonoda et al., 1997). In our study, we found that staurosporine in higher concentrations inhibited expression of Gro (CXCL1). Defining the inhibitory effect of staurosporine to specific pathways can not be performed due to lack of specificity. It is possible that due to its very non-specificity, staurosporine affects other genes that may be indirectly involved in chemokine gene expression.

Staurosporine has been reported to inhibit NF-κB activation (probably by inhibition of IkB kinases) and has been shown to inhibit pro-inflammatory cytokines (TNF-α, IL-6 and IL-1) signalling outcomes and control the expression of chemokines (Feng and Kaplowitz, 2002). In conclusion, these in vitro data may aid to a better understanding of the pathways in chronic liver injuries and identify clinical studies that may aid in treatment or prevention of these conditions.

Furthermore, there may be further potential to prevent changes to hepatocyte phenotype and allow isolation of hepatocytes with a greater physiological phenotype.

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