Expression of regulated oncogen-alpha by primary hepatocytes following isolation and heat shock stimulation

Gholamhossein Hassanshahi[1*], Mohammad Ebrahim Rezvani[2], Mohammad Kazemi Arababadi[1], Ali Shamsizadeh[2], Mehdi Mahmoodi[3], Ali Mousavi[4], Alan James Dickson[5]

[1]Department of Hematology and Immunology, School of Medicine, Rafsanjan University of Medical Sciences, P.O. Box 77175-835, Rafsanjan, I.R. Iran [2]Department of Physiology, School of Medicine, Rafsanjan University of Medical Sciences, P.O. Box 77175-835, Rafsanjan, I.R. Iran [3]Department of Biochemistry, Ail Ebne Abitaleb Teaching Hospital, Rafsanjan University of Medical Sciences, P.O. Box 77175-835, Rafsanjan, I.R. Iran [4]Department of Emergency, Ail Ebne Abitaleb Teaching Hospital, Rafsanjan University of Medical Sciences [5]Department of Biochemistry, Faculty of Life Sciences, University of Manchester, UK

Abstract

High levels of regulated oncogen-alpha (GRO-α) expression have been observed in the liver. GRO-α stimulates proliferation of epithelial cells and induction of rolling and extravascular migration of neutrophils and mononuclear cells. Given the above observations, this chemokine was chosen to be analyzed in freshly isolated and cultured hepatocytes. In this study, hepatocytes (2×10^6 cell/ml) were isolated from male Sprague Dawley rat liver and cultured on plates that were pre-coated with collagen type-I matrix. The western and northern blot analyses were employed to detect GRO-α at the protein and mRNA levels in freshly isolated and cultured hepatocytes in response to isolation and heat shock stresses. GRO-α was shown to be expressed by isolated rat hepatocytes immediately after isolation and early culture and decreased with time. mRNA was also expressed in freshly isolated cells (0 h) and did not decrease after 48h of culture and further time points ($P<0.01$). These results also demonstrated that expression of GRO-α by hepatocytes increased in response to heat shock at different time points in comparison with the control ($P<0.01$). These results demonstrated that the isolation and heat shock stresses induced the expression of GRO-α in hepatocytes in a time-dependent manner. Thus, it seems that hepatocytes mimic the experiences that the liver encounters after injury *in vivo*. In such a situation, liver produces stress related agents like chemokines to overcome injurious conditions.

Keywords: Hepatocyte; GRO-α; Chemokine; Heat shock

Correspondence to:* **Gholamhossein Hassanshahi, Ph.D.
Tel: + 98 391 5224003-4; Fax: + 98 391 5225209
Email: ghassanshahi@gmail.com

INTRODUCTION

Chemokines are low molecular weight proteins (8-17 kDa), which are classified into four distinct groups named CXC, CC, CX3C and C. Depending on the presence or absence of the ELR (Arg-Leu-Glu) motif before the first cysteine residue in their structures, the CXC chemokines are also subdivided into ELR+ and ELR-. GRO-α will fit into the category of ELR-CXC chemokines (Hassanshahi *et al*., 2007a; Hassanshahi *et al*., 2007b; Varley *et al*., 2003).

GRO-α is a CXC chemokine that was originally described as having melanoma growth stimulatory activity and was subsequently shown to activate human neutrophils (Richmond and Thomas, 1988; Wang and Richmond, 2001), however, two additional GRO-α products, GRO-α-β and GRO-α-γ, were later discovered (Haskill *et al*., 1990). The murine homologue of GRO-α is the KC gene that has been cloned from mouse fibroblasts as a platelet derived growth factor (PDGF)-inducible gene. The CINC (cytokine induced neutrophil chemoattractant) as the rat homologue of GRO-α was also cloned from rat macrophages. GRO-α stimulates proliferation of epithelial cells (Suzuki *et al*., 2000) and induction of rolling and extravascular migration of neutrophils and mononuclear cells (Olszyna *et al*., 2001; Zhang *et al*., 2001). Monocytes, neutrophils, endothelial cells, fibroblasts, skin keratinocytes, melanoma cells and hepatocytes (Shiratori *et al*., 1994) have been shown to produce GRO-α (Baggiolini *et al*., 1997).

In the process of isolation and culture, some stress-

related signals will be activated, leading hepatocytes to enter a response similar to the stress response that occurs in immune responses, characterized with early expression of some mediators including chemokines to overcome the injurious situation. Expression of GRO-α in hepatectomized mouse liver (Su *et al*., 2002) and rat H4 hepatoma cells (Hassanshahi *et al*., 2006) has been demonstrated and may play an important role in hepatocyte proliferation and liver regeneration.

Increased GRO-α expression is associated with some hepatic injuries and diseases, including liver ischaemia/reperfusion in mice and rats (Moser *et al*., 1991; Hisama *et al*., 1996; Kobayashi *et al*., 2002), infection, paracetamol poisoning and sepsis following LPS injection in mice (Salkowski *et al*., 1998; Hogaboam *et al*., 1999; Mercer-Jones *et al*., 1999). In humans, elevated levels of GRO-α have been shown in alcoholic hepatitis (Maltby *et al*., 1996).

This work aimed to clone and analyse the expression of GRO-α at the level of RNA and protein by hepatocytes during their isolation and early and also examine the effects of heat shock stimulation on expression of the GRO protein.

MATERIALS AND METHODS

Perfusion, isolation and maintenance of hepatocytes in culture: As mentioned in previous researches (Seglen, 1976; Varley *et al*., 2003), briefly, hepatocytes from fed male Sprague-Dawley rats weighing approximately 200 g, were isolated by perfusion of the liver with Krebs-Henseleit bicarbonate solution (128 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$) and collagenase (Sigma-Aldrich, UK) under aseptic conditions. After 10 min, the liver was removed under sterile conditions and gently broken down and filtered through an sterile gauze with Krebs-Henseleit bicarbonate solution. In order to harvest a population of large parenchymal cells, the cells were washed three times by differential centrifugation and gently resuspending the pellets in Krebs-Henseleit bicarbonate solution at each time. 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 more than 85% viable; these were generally 90-95% viable. The hepatocytes were of high purity and when observed 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 staining with specific antibodies against the von Willebrands factor (Santa Cruz Biotechnology, California, USA). The hepatocytes were seeded (2×10^6 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% CO_2 in O_2. After 3h in culture, the culture media was replaced with maintenance medium [Waymouths MB/721 media supplemented with BSA (bovine serum albumin, 0.2% w/v) and sodium oleate (0.0005% w/v)].

Heat shock stimulation: For the purpose of heat shock stimulation, hepatocytes were isolated from male Sprague Dawley rat liver. The cells ($2\text{-}5\times10^6$) were then plated onto culture plates that were pre-coated with collagen type-I matrix. Cells were suspended in inoculation medium and then added to culture plates. Cultured cells were incubated in an atmosphere of 5% CO_2: 95% O_2 at 37°C for 3 h. After 3 h of incubation, in the case of the heat-shock treatment, medium was replaced with fresh inoculation medium and plates containing cells were wrapped in a parafilm and placed in a pre-heated water bath (42°C) for 20 min and then normal incubation was continued for 3h, 6h and 24h. Control cells were assessed for each time point in parallel with the heat-shock treated cells. Medium was removed at indicated time points, centrifuged and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The immunoblotting data were quantified by densitometry analysis.

Cloning: An invitrogen TA cloning® kit (Invitrogen, USA) with a pCR®2.1 vector was used for cloning of the amplified cDNA produced from RNA. The following reaction was set up: 1 µl of fresh polymerase chain reaction (PCR) product, 1 µl of 10 X ligation buffer, 2 µl of the pCR®2.1 vector (25 ng/µl stock concentration), 1 µl of T4 DNA ligase (4.0 Weiss units) were mixed and 5 µl of sterile double distilled water was added to give a final volume of 10 µl. The ligation reaction was incubated overnight at 14°C. Vials containing the ligation reactions were pulse-centrifuged and placed on ice. 2 µl of each ligation reaction was pipetted directly into a vial of competent cells (TOP10, supplied with the kit and kept on ice) and mixed by stirring gently with the pipette tip. The vials were incubated on ice for 30 min and the remaining ligation mixtures were

stored at -20°C. The vials were heat-shocked for exactly 30 seconds in a pre-warmed 42°C water bath and then placed on ice. 250 µl of SOC medium (supplied with the kit and pre-equilibrated to room temperature) was added to each tube and the vials were shaken horizontally at 37°C for 1 h in a shaking incubator. 50 µl from each transformation vial was spread onto separate LB agar plates pre-warmed at 37°C and containing 40 µl of X-Gal (X-galactose, 40 mg/ml) and 50 µg/ml of ampicillin. Once the liquid was absorbed, the plates were inverted and incubated at 37°C overnight. The plates were then incubated at 4°C for 2-3h to allow for proper color development. The TOPO vector used contains a *LacZ* gene that codes for β-galactosidase. The enzyme cleaves the colorless X-gal, forming a blue precipitate. This gives an efficient selection method as the multiple cloning site, where the PCR product inserts, interrupts the *LacZ* gene rendering it non-functional. The overnight plates therefore contain blue and white colonies, the white ones being the ones that may contain plasmid with inserts.

Using aseptic techniques, 10 white colonies were picked for plasmid isolation and restriction analysis and grown overnight in 10 ml of LB broth containing 50 µg/ml of ampicillin. In addition two blue colonies were also picked as controls to confirm that they did not contain the insert. From the mentioned overnight culture plates, one colony of the transformed cells was selected to inoculate 10 ml of LB medium containing ampicillin. This culture was incubated at 37°C and allowed to grow overnight with shaking. 5 ml of the overnight culture was then added to 95 ml of LB media containing ampicillin and was incubated overnight as above. After this incubation the suspension was centrifuged at 15000 ×*g* for 15 min at 4°C. The PCR®2.1 fragment-containing plasmids were then isolated from the cells using a Qiagen plasmid midi preparation kit (invitrogen, USA). At the first stage of this isolation the bacterial cell pellet was re-suspended in 4 ml of P1 buffer (pH 8) (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml of RNAase A). 4 ml of P2 lysis buffer containing 200 mM NaOH and supplemented with 1% (w/v) SDS was then mixed slowly and gently and the resulting mixture was then incubated at room temperature for 5 min. After this period, 4ml of ice-cooled P3 neutralization buffer (3 M potassium acetate, pH 5.5) was added and the solution was further incubated for 15 min on ice. Centrifugation was performed at 20,000×*g* for 30 min at 4°C. The supernatant was removed and again centrifuged for another 15 min. Finally the supernatant was added to a Qiagen tip-100 column, previously washed with 4 ml of QBT buffer (pH 7) (750 mM NaCl, 50 mM MOPS, 15% (v/v) ethanol, 0.15% (v/v) Triton X-100). The column was then washed twice with 10 ml of QC buffer (pH 7) (1 M NaCl, 15% (v/v) ethanol, 50 mM MOPS). The plasmid DNA was eluted using 5 ml of QF buffer (pH 8.5) (1.26 M NaCl, 15% (v/v) ethanol, 50 mM Tris-HCl). Subsequently the plasmid DNA was precipitated by addition of 3.5 ml of isopropanol and then centrifuged at 15,000 ×*g* for 30 min at 4°C to obtain the pellet. To obtain a better purity and qualitative recovery, the plasmid DNA pellet was re-suspended in 2.5 ml of 70% (v/v) ethanol and re-centrifuged. The obtained pellet was allowed to air-dry for 5-10 min and dissolved in 25 or 50 µl (depending on the size of pellet) of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). To measure the DNA concentration and quality of DNA in plasmid preparations, each sample was diluted 1:200 in double distilled water and the absorbance of the sample was read at 260 nm and 280 nm in a quartz cuvette (Japan). The reading was standardized against double distilled water. The ratio of absorbance, 260 nm/280 nm was used as an indicator of purity of DNA with a ratio ranging from 1.6 to 2.0 showing a relatively pure preparation of plasmid. An absorbance of 1.0 at 260 nm is equal to 50 µg of DNA/ml.

To digest the fragments from recombinant PCR®2.1, plasmid DNA (5 µl from minipreps or 1-5 µg from midipreps) was mixed with 2 µl of the appropriate 10X buffer, 1 µl of appropriate restriction enzyme and, in the case of the minipreps, 1 µl of RNase A (25 µg/µl, pre-boiled for 10 min) and diluted to a final volume of 10 µl with double distilled milli Q water. The mixture was then incubated for 1 h at 37°C. The results of this digestion were analyzed by agarose gel electrophoresis. The digestion product was mixed with 1 µl of DNA loading buffer (1 mM EDTA, 50% (w/v) glycerol, 20% (w/v) ficoll, 4% (w/v) bromophenol blue). The products were separated on a 1% (w/v) agarose TBE gel (1g of agarose, 90 ml of double distilled water, 10 ml of (10X) TBE buffer, 0.5 µl of a 5 mg/ml solution of ethidium bromide). TBE buffer (10X) consisted of 0.9 M Tris-HCl, 0.9 M orthoboric acid and 0.04 M EDTA, and was used as running buffer at 1X concentration. A λ 1kb DNA ladder (Invitrogen, USA) was used as a reference to estimate the size of the unknown DNA fragments. Electrophoresis was performed for 30-60 min at 70V. A UV-transilluminator (white/UV transilluminator, UVP, USA) was used to visualize the DNA bands.

Following restriction digestion and gel separation,

the appropriate fragments were excised using a sterile scalpel. The excised piece of gel, containing the desired DNA fragment, was weighed and its volume was calculated assuming that 0.1 g of gel was equal to 100 µl. 4.5 volumes of NaCl solution and 0.5 volumes of TBE modifier (both supplied with the GeneClean III® kit) were added to the gel. The mixture was heated to 55°C, and allowed to solubilize. An appropriate volume of EZ-Glassmilk (Defatted milk, marvel^{tm}, UK) was added to the extracted gel mixture and, after mixing, was incubated for 5-10 min at room temperature. This slurry was centrifuged at 10,000 × *g* for 15 s (at room temperatures). The supernatant was discarded and the pellet was re-suspended in 200 µl of New Wash solution (Qiagen Ltd, UK) and centrifuged as before (this stage was repeated twice). The pellet was harvested and, after washes, was air-dried for 5 min and 25 µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8) was added and the suspension was centrifuged at 10,000 × *g* for 30 s at room temperature. The supernatant (containing the DNA fragment) was transferred to a fresh sterile microcentrifuge tube and stored at -20°C. The recovery of the purified fragment was confirmed by running a small volume of the final supernatant on a 1% (w/v) agarose gel.

Northern blot analysis: For the purpose of northern blotting, the electrophoresis tank was set up in the fume hood and a gel was prepared by mixing the following reagents: 10 ml 10X MEA [MOPS (3-N-morpholino-propanesulphonic acid), EDTA and acetate buffer, pH 7], 75 ml of DEPC (diethyl polycarbonate) treated water and 1g of multipurpose agarose. The solution was heated in a microwave until the agarose dissolved. The gel was allowed to cool until hand-hot and 15 ml of 37% (v/v) formaldehyde was added. The mixture was poured into the gel tank and allowed to set. For each sample to be analyzed, 20 mg of RNA was made up to a final volume of 20 ml with DEPC-treated water. Denaturing buffer was prepared by mixing 20 ml of 10X MEA buffer (pH 7.0), 35 ml of formaldehyde and 100 ml of formamide and 10 ml (minimum) of this mixture was added to each sample. The mixture was pulse-centrifuged and heated for 15 min at 65°C and pulse-centrifuged again to collect the entire mixture at the bottom of tube.

The samples were mixed with 2 ml of RNA loading buffer [(1 mM EDTA, 50% (w/v) glycerol, 4% (w/v) bromophenol blue 0.25% (w/v), pH 8.0] and were loaded onto the gel. The gel was immersed in running buffer (1X MEA buffer pH 7.0) and electrophoresis was performed at 70-80 V until the dye front was about half the way down the gel. The gel was placed in a sandwich box with 250 ml of DEPC-treated water supplemented with 1 mg/ml of ethidium bromide. This was shaken very gently and slowly at room temperature for 15-20 min. The liquid containing ethidium bromide was removed. Then the gel was de-stained in DEPC-treated water with gentle shaking at room temperature and the presence of RNA was visualized by UV transillumination and photography. Before transfer to nylon membrane, the gel was soaked in 10X SSC (1.5 M NaCl, pH 7.0, containing 0.15 M Tri-sodium citrate) for 20-30 min and placed in a capillary transfer apparatus. The system was left overnight to complete transfer. Then the membrane was removed and dried for 20-60 min. UV light was used to confirm the transfer of RNA species from the gel to the membrane. Defined 18S and 28S rRNA bands indicated intact RNA and the position of these bands and lanes were marked by pencil. The membrane was baked for 2h at a pressure of 20-25 mmHg and stored at room temperature until use. The oven was warmed to 65°C and membrane was placed in a hybridization tube to which 10 ml of pre-hybridization solution (0.5 M Na_2HPO_4, pH 7.2, containing 1 mM EDTA, 7% (w/v) SDS and 1% (w/v) BSA) was added and left to incubate for 1-4h at 65°C. Between 50-100 ng of all cDNA probes (except for 18S rRNA, see below) were radioactively labeled with ^{32}P-adATP or ^{32}P-adCTP using a random primed kit according to the manufacturer's instructions (the reaction was performed at 37°C and was stopped by addition of 2 ml of 0.2 M EDTA (pH 8.0).

Unincorporated nucleotides were separated from the radioactive DNA probe through a Sephadex G-50 spun column. Sephadex columns were produced by plugging of a 1ml syringe with siliconized glass wool and filling the syringe with 15% (w/v) Sephadex G-50 (Sigma, USA) dissolved in TEN buffer (10 mM Tris (pH 8.0), 0.1 M NaCl and 1mM EDTA). 100 ml of TEN buffers was added to the column and the column was placed in a 15 ml Falcon tube and centrifuged at 250 g for 1 min at room temperature. More reaction mixture and TEN buffers were added and centrifugation was continued until a 1 ml total column of concentrated Sephadex G-50 was achieved. The radioactive reaction mix [fragment (1 ml) and up to 10 ml of water], 1ml of dGTP, 1 ml of dCTP, 1 ml of dTTP, 2 ml of reaction buffer, 5 ml of radioactive ^{32}P-adATP or ^{32}P-adCTP, 1 ml of Klenow transcription enzyme and 100 ml of TEN buffer were added to the column and centrifuged as above. The eluted

purified probe was incubated for 5 min at 100°C and then cooled on ice for 5 min.

The 18S cDNA probe was labelled using a Nick Translation kit (Boheringer-Mannheim group Ltd, UK), according to the manufacturer's instructions. The reaction mixture was incubated at 15°C for 30 min and then stopped by the addition of 2 ml of 0.2 M EDTA (pH 8.0). The probe was then boiled for 5 min and then placed on ice for another 5 min prior to the addition to the pre-hybridized membrane. The pre-hybridization solution was removed. The probe that was generated was then added to 15 ml of fresh hybridization solution (0.5 M Na_2HPO_4 (pH 7.2) containing 1 mM EDTA, 7% (w/v) SDS and 1% (w/v) BSA) and this mixture was added to the appropriate pre-hybridized membrane for incubation at 65°C overnight. After overnight incubation, the membrane was washed at 65°C for 15 min with 15 ml of wash buffer (40 mM Na_2HPO_4 (pH 7.2) containing 1 mM EDTA, 5% (w/v) SDS and 0.5% (w/v) BSA). Two, or if need be, three washes were performed at 65°C for 15 min with 15 ml of another wash buffer (40mM Na_2HPO_4 (pH 7.2) containing 1 mM EDTA, 1% (w/v) SDS). The number of washes was dependent on the level of radioactive background that was detected on the membrane by a hand-held radioactive monitor. After washing the membrane was sealed in a plastic bag and exposed to a phosphor image plate (Kodak, USA) for 1-4h. To make a permanent copy of the results, the membrane was exposed to X-ray film in a cassette with an intensifying screen (Kodak, USA). Exposure was performed at -80°C. X-ray film was developed with a M35-M X-OMAT processor (Kodak, USA). For stripping the, membranes were placed a sandwich box and 200 ml of stripping buffer (0.1% SDS (w/v) in distilled water) was pre-boiled and added to the membrane. Membranes were left for 2-3 hours at room temperature with gentle agitation, and then sealed in a plastic bag and left for 2-3 days to ensure that they stripped perfectly and bands did not appear. Membranes could then be re-probed.

Western blot analysis: At indicated time points, medium was removed from hepatocyte cultures and centrifuged. Clarified supernatants from the 0h and incubated samples were used for SDS-PAGE. The freshly isolated hepatocyte culture medium supernatant at the 0h time point and the untreated hepatocyte culture medium at each time point of heat shock stimulation were used as controls in the study. Immunoblotting and densitometry were performed to quantify the expression of stormal derived factors-α1 (SDF-1). Equal amounts of protein (35 μg) were loaded and resolved on a 10% (w/v) SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. After blocking with 3% (w/v) milk in phosphate buffer 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 GRO-α (Chemokine.com, Houston, USA, supplied by AMS Biotechnology, Abingdon, UK). Subsequently, anti-rabbit horseradish peroxidase-conjugated antibodies (sigma, USA) (72 pg/μl) were used accordingly and the ECL detection system (Amersham International) was used to define protein localization and quantity.

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 GRO-α was selected for detailed examination and western and northern blotting methods were applied to assess the expression of this CXC chemokine.

The cloning and sequencing of GRO-α gene fragment: The cloning and sequencing of GRO-α gene fragment are demonstrated in Figures 1 and 2. The molecular size of GRO-α gene fragment was approximately 1100 bp which was consistent with other previous observations. The sequencing of GRO-α gene fragment is shown in Figure 2. The pattern of the sequenced fragment is exactly identical to the previously sequenced fragment available in the Pubmed nucleotide database (Accession number: NMO22177).

Expression of GRO-α during basal culture of hepatocytes: *Protein level*: As shown in Figure 3, the GRO-α protein was highly expressed in freshly isolated hepatocytes (0h). In other words, the expression of GRO-α protein sharply increased immediately after hepatocyte isolation. However, it was observed that the expression of GRO-α protein significantly decreased with progression of the time up to 30h after isolation and then remained steady up to 80h.

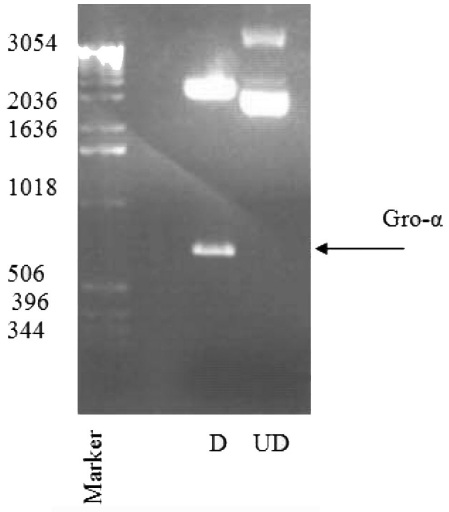

Figure 1. Restriction digest of pCR®2.1 plasmid containing cDNA. EcoRI and ClaI digestion of the pCRÒ2.1 plasmid vector containing cDNA amplified using primer sequences for GRO-α. An Invitrogen TA cloningÒ kit was used for insertion of the PCR product into the plasmid. The vector was then transformed into Oneshot® cells (TOP10) and a mini-preparation was performed. Fragments were resolved on a 1% (w/v) agarose gel. D=Digested and UD= Undigested. Numbers on the left show the sizes of the bands of the DNA molecular size markers.

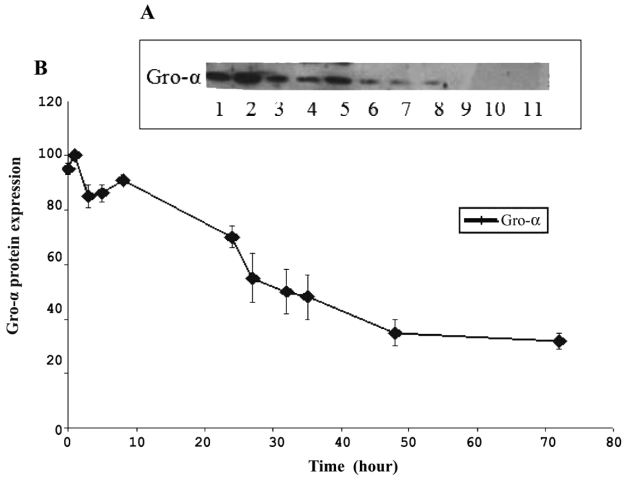

Figure 3. Expression of GRO-α at the protein level by hepatocytes during primary culture. A) Representative profile of protein bands from western blotting of GRO-α expression following hepatocytes' isolation and early culture. Lane 1=0h (freshly isolated hepatocytes used as control); lane 2=1h; lane 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 GRO-α protein expression following hepatocytes' isolation and early culture. The expression of GRO-α protein sharply increased immediately after isolation and then significantly decreased with time. [*P< 0.05 v 100% value]. The densitometry of bands (6 densitometric replicates) on the X-ray film was determined by densitometric analysis using a Model GS-700 Imaging Densitometer and Molecular Analyst® software.

mRNA level: As shown in Figure 4, the expression of GRO-α at the mRNA level is parallel to the pattern of protein expression. The expression of mRNA markedly increased in fresh isolated hepatocytes, with maximal expression being observed at 0h. However, GRO-α mRNA expression decreased with advancing time, but then reduced significantly at 24h of culturing.

Analysis of expression of GRO-α protein in response to heat shock: As indicated in Figure 5, heat shock had stimulated expression of GRO-α, and a 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 incubation times progressed (up to 24h).

ttcacttcaagaacatcca gagtttgaaggtgatgccgccaggaccccactgca *1**ccnantgtaccnaaac cgnnnccatagccacactcaagaatggtcgcgaggcttgccttgaccctgaagcccccatggttcag aagattgtccaaaagatgctaaagggtgtccccaagtaatggagaaagaagatagattgc accgatgg cgtcngtctggtgaacgctggcttctgacaacactagtt ntacacatttacgatttctattgagggtc** ctat ttattttatgtatttatttattccaccaagtgtgtggtttttatttta *2**cattaatatttaacgatgtggatgcgtttcatcg atggtcgttcaattccaattgtgcagtttaaagatggtaggcgttaaatatctcgttaaattaatatttattgg gagaccattaggt atcaaccactgtgctagaaggtgttgagcgggaagaagggcggagagatgaga gtctggga tcgtgttttg agttagggtgaggaat atgtgagaggctatg ntgtatgttttg naagaatgtta tttattgaaagttgtctttcatattttatggtcaacattgatgtgttgaagct** tcccttggacattttatgtctagtttgt agggcacaatgcct tttatattctttaaccaatgctc

Figure 2. Sequencing data for GRO-α. Sequence of rat GRO-α (M86536) has been taken from the nucleotide database (NCBI). The sequences of the primers used for amplification are shown in the boxed region. Numbers denote successfully the sequenced regions 1 and 2 (bold area). *1(sequenced region 1) shows identities for region 1=208/221 (94%). *2 (sequenced region 2) shows identities for region 2= 297/302 (98%). Underline bases denote differences between sequencing data and those of the database.

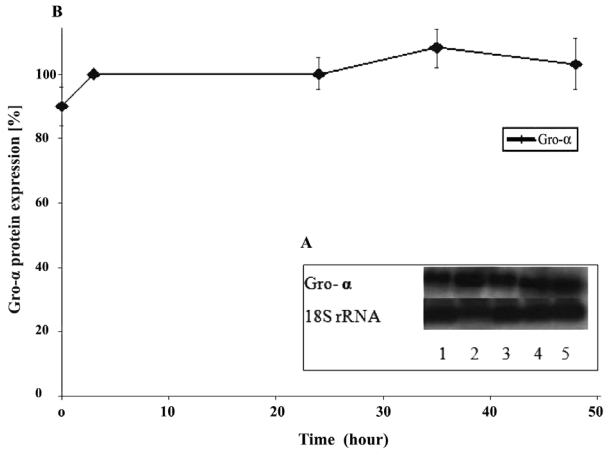

Figure 4. Expression of the GRO-α at the mRNA level by hepatocytes during primary culture. A) A representative northern analysis of GRO-α mRNA/rRNA ratio probed with [_-32 p] dCTP-labelled IP-10/Mob-1, cDNA. 18S rRNA was used for loading of equal amounts of RNA in the well. Lane 1=0h; lane 2=3h; lane 3=24h; lane 4=35h; lane 5= 48h. B) Time-dependent variation in GRO-α mRNA expression following hepatocytes' isolation and early culture. The expression of GRO-α mRNA sharply increased following hepatocytes isolation and decreased with time.[*P< 0.01 v 100% value]. The densitometry of bands (6 densitometric replicates) on X-ray film was determined by densitometric analysis.

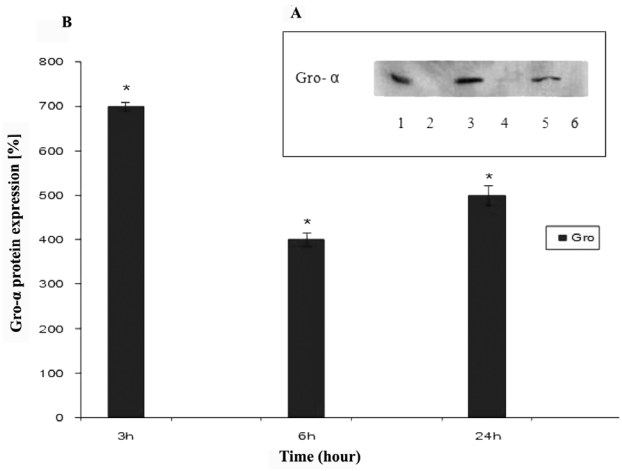

Figure 5. Expression of the GRO-α at the protein level in response to heat shock. A) Representative profile of GRO-α protein expression following heat shock induction. Lane 1=3h after heat shock treatment; lane 2=3h control; lane 3=6h after heat shock treatment; lane 4=6h control; lane 5=24h after heat shock treatment; lane 6=24h control. The control bands are indicated in lanes, 2, 4 and 6. B) Time-dependent variation in expression of GRO-α protein following heat shock stress on the hepatocyte 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]. The densitometry of bands (6 densitometric replicates) on X-ray film was determined by densitometric analysis.

DISCUSSION

Although the designed primers were expected to be specific for cloning of the CXC chemokines GRO-α and the cloned fragments released in digests were the right size when compared with molecular markers, the identity of cDNA fragments was again confirmed by sequencing of cloned products (Fig. 3). Figure 3 shows nucleotide sequences of the regions that were successfully sequenced. Only a partial sequence was obtained for GRO-α, which was significantly identitcal with the database sequences of GRO-α (96%), thus verifying that the sequences obtained in the plasmids were those of the relevant CXC chemokines. GRO-α, cDNA species were used as probes in northern blotting analysis. As is obvious from Figure 4, GRO-α mRNA was expressed in freshly isolated cells and remained relatively high and constant for 48h (Fig. 4). Chemokine cDNA fragments produced by RT-PCR reactions were of the expected size. Furthermore, the fidelity of these fragments was confirmed by DNA sequencing analysis, indicating that they were suitable for use as probes in northern blotting analyses. In this study, expression of ELR+ GRO-α was analysed at the mRNA level using northern blot analysis. There is clear evidence from our study to indicate that GRO-α is produced by isolated hepatocytes and that, in some cases, there is an activation of expression at the mRNA level as a result of cell isolation and culture. Although, induction of the chemokine gene expression in macrophages, fibroblasts and osteoblasts is regulated by both transcriptional and post-transcriptional and cell type-dependent pathways (Ohmori and Hamilton, 1994), the mechanisms that regulate chemokine gene expression in hepatocytes are yet to be fully identified. GRO-α expression occurred immediately after isolation of hepatocytes and continued for up to 48 h. 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) More recently in another study it was shown that other CXC chemokines such as SDF-1 and interferon gamma inducible protein-10 (IP-10) also has been expressed by hepatocytes following isolation and heat shock

stimulation (Hassanshahi *et al*., 2007a; Hassanshahi *et al*., 2008).

It would be of interest to carry out a comparative review of what is known of promoter regulatory elements for GRO-α. GRO-α (Ohmori and Hamilton, 1995) has been shown to have consensus response elements for the binding of nuclear factor-kappa B (NF-κB). In fact the NF-κB pathway may allow for a mechanism to specifically activate expression of these three chemokines specifically. In addition to the NF-κB, the presence of AP-1 and nuclear factor-interleukin-6 (NF-IL-6) consensus also has been shown in GRO-α promoter.

Finally, angiogenic roles have been suggested for GRO-α (Luster *et al*., 1995; Xiong *et al*., 2004), and the expression of this chemokine for longer periods may indicate a role for GRO-α in angiogenesis following hepatic injury. Heat shock activates signaling pathways with very notable changes in JNK and p38 (protein 38) isoforms and their upstream activators like MKK3 and MKK6 (MAP kinase kinase 6) (Maroni *et al*., 2000). Consequently heat shock offers a useful mimetic stimulus for comparison with the effects of cell isolation. In the present study, GRO-α was shown to exhibit 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 (MAPKAP-2) (Zhang *et al*., 2004).Thus the expression of various HSPs, (e.g, HSP 25, 27, 60, 70 and 90) has been shown to occur in the liver in response to heat shock. HSP70 expression increases as a result of hepatectomy and has been observed in proliferating hepatocytes (Ohmori and Hamilton, 1995), whilst HSP25 expression is elevated in liver carcinomas (Diehl, 2000). The presence of a heat shock response element has been demonstrated in the promoter of some CXC chemokines such as (interferon-inducible protein 10) IP-10 but it is absent from the GRO-α promoter. Thus, it is unclear how the expression of this protein is increased in the heat shock studies of this investigation. The heat shock stimulation may modulate some other unknown regulatory elements in this gene or produce secondary effects through other regulatory genes (potentially from pro-inflammatory cytokines to growth factors) and indirectly induce expression of GRO-α.

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