Assessment of NK Cells Response to Hepatocyte Derived Chemotactic Agents

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Abstract: This project was aimed to examine the NK92 cells response as the CXC chemokine responder cells in rat model of liver disorder and injuries. Hepatocytes were isolated from Sprague-dawley rats and cultured on collagen type I. Migration of NK92 cells was assessed using a 48 well micro-chemotaxis technique. Transwell chambers were positioned face up, blocked Medium supernatant (500 µL) obtained from hepatocytes cultures were placed into the lower compartment of each Transwell. The upper compartment was filled with either 500 µL of NK92 cells. After washing, Membrane-attached cells were fixed; stained and Membrane-attached cells were counted by light microscopy and/or by size gating (9-14 μm) with an automated counter system. Human NK92 cells were attracted to recombinant human IP-10 in a concentration and time-dependent manner. NK92 cells also exhibited a chemotactic response to medium harvested from primary hepatocyte cultures. Isolated and cultured hepatocytes express several different chemokines. Although we identified that medium from hepatocyte cultures contains specific chemokines by immunoblotting, there is potential that migration assays detected yet other chemokines and other factors such as complement components. In this report, we demonstrated that hepatocytes expressed factors that were chemoattractive for human NK92 cells and that the factors must interact with the repertoire of receptors responsible for recruitment of these cells.

Key words: CXC chemokine, NK92 cells, chemoattractive

INTRODUCTION

Several known injurious conditions such as hepatitis, endotoxemia/reperfusion, sepsis/endotoxemia and drugs cause recruitment of macrophages, neutrophils and other immune cells to the liver (Jaschke, 2002). Although neutrophils and Kupffer cells are widely considered to be the first defenders of liver, the liver immune response is not exclusive to these cells and other immune cells (such as T lymphocytes and NK cells) are also involved in these processes and in most part. Involvement of CC chemokines such as MCP-1, ELR CXC chemokines (such as IL-8/MIP-2) and ELR-chemokines (such as IP-10/Mob-1) (Koniaris et al., 2001; Ishida et al., 2002) in acetaminophen-induced liver injuries and modulation of liver regeneration processes, has been reported by Ren et al. (2003). Increased concentration of IL-8/MIP-2 has been documented in conditions of liver transplantation, ischemia/reperfusion, alcoholic hepatitis and fibrotic liver (Kobayashi et al., 1999). High constitutive levels of SDF-1α have been observed in the non-inflamed biliary epithelium of the liver (Coulomb et al., 1999) in association with CXCR4 expressing lymphocyte recruitment (Terada et al., 2003). 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 via the biliary tract (Mitra et al., 2001). Enhanced expression of 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 but high expression of IP-10 has been shown to occur in response to hepatic injury and partial hepatectomy (Koniaris et al., 2001), alcoholic liver disease (Kobayashi et al., 1999) and liver sepsis (Salkowski et al., 1998). Hepatocytes in patients suffering from chronic hepatitis produce IP-10 and the concentration of IP-10/Mob-1 is increased in serum of patients with cirrhosis, chronic B, C and autoimmune hepatitis (Shields et al., 1999; Kakimi et al.,

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Several independent research groups have documented the stimulation of expression of IP-10/Mob-1 subsequent to hepatocyte isolation (Hassanshahi et al., 2007a). Neutrophils and granulocytes express CXCR1, CXCR2 and CXCR4 (Baggiolini, 2001). CXCR3 and CXCR4 has been demonstrated to be expressed on lymphocytes (Th1 and Th2), monocytes and NK cells, leukemic B cells, plasmacytoid and myeloid cells and dividing microvascular endothelial cells (Romagnani et al., 2002). Hence this project aimed to examine the NK92 cells response as CXC chemokine responder cells in rat model of liver disorder and injuries.

**MATERIALS AND METHODS**

**Perfusion, isolation and hepatocytes culture:**
Hepatocytes were isolated 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 8-10 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 90-95% 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 (2×10⁶ cell mL⁻¹) onto to 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 (0.005% w/v)]. The cells were treated as described in the figure legends.

**In vitro NK92 cells culture:** The human cell line NK92, were used to assess functional CXC chemokines as part of chemotaxis assay. The NK92 human cell line was a generous gift from Dr. H. Young (National Institute Frederick USA). The cell line was grown in suspension in a culture medium consisting of RPMI 1640 supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 100 U mL⁻¹ Penicillin and 100 µg mL⁻¹ Streptomycin. To ensure optimum growth conditions for the cell line, culture flasks were checked regularly and the medium was replaced in every third day. To initiate a chemotaxis assay, a total cell count was performed (Casy I cell counter + analyzer system model SCAREF System GmbH) and cells were adjusted with medium to give a density of 1-2 mL⁻¹×10⁶ in 25 cm² tissue culture flasks. Medium supplemented with 200 IU of IL-2 mL⁻¹ and 10 ng of IL-15 mL⁻¹ was added to the culture and 18 h later the medium was removed and replaced with medium freshly supplemented with IL-2 and IL-15 and a chemotaxis assay was performed.

**In vitro chemotaxis assay:** Migration of peripheral blood leukocytes and NK92 cells was assessed using a 48 well micro-chemotaxis technique. The two compartments were separated with a polycarbonate (polyvinylpyrrolidone-free) filter (6.5 µm pore size Corning Incorporation). Transwell chambers were positioned face up, with the membranes pre-coated with fibronectin (10 µg mL⁻¹ for 1-3 h in an atmosphere of air: 5% CO₂ and 37°C). After incubation with fibronectin, the fibronectin solution was removed and the chamber membrane was blocked (for 30-45 min) with heat-inactivated BSA (10 mg mL⁻¹ BSA in PBS, after treatment at 85°C for 13 min). Membranes were subsequently washed twice with PBS to eliminate residual BSA. Medium supernatant (500 µL) obtained from hepatocytes culture were placed into the lower compartment of each Transwell. The upper compartment was filled with either 500 µL of human peripheral white blood cells or NK92 cells (0.5×10⁶ peripheral white blood cells or 0.5×10⁹ NK92 cells) suspended in their appropriate culture medium. Cultures were continued at 37°C in an atmosphere of air: CO₂ for various periods. At this stage two different fractions of cells were assessed, one fraction being the cells that were attached to the polycarbonate membrane and the second fraction was the cells that passed through the membrane into the lower chamber (passed cells). At the end of incubation, membranes, with attached cells, were washed 3 times with PBS. Membrane-attached cells were fixed by addition of methanol to the membranes (for 2-5 min) and then washed 3 times with PBS. Fixed membrane-attached cells were stained either with crystal violet [0.1% (w/v) in double distilled water] for 15-20 min or with Jenner-Giemsa’s. For staining of the fixed membranes using Jenner-Giemsa’s method, membrane were stained first in Jenner solution [a solution of (0.3% (w/v) Jenner stain, in 70% methanol) for 2 min and then by addition of an equal...
portion of PBS (pH 6.4) for a further 1 min. The Jenner solution was removed and Giemsa’s stain [Giemsia’s stain 2% (v/v)], in PBS (pH 6.4) was added for 30 min and at the end of incubation membranes were washed by three changes in PBS (pH 6.4) and air-dried. Membranes were cut and mounted for further analysis. Membranes were mounted by using one drop of Loetite 357 resin was placed onto the membrane, then, a coverslip was applied and firmly pressed down. A permanent mount was achieved by exposing the membrane to ultraviolet light from a lamp held in close proximity to the slide for about 30 sec. Membrane-attached cells were counted in 3 high power fields (400X) under light microscopy, after coding the samples. Membrane-permeant cells were counted by light microscopy and/or by size gating (9-14 μm) with an automated counter system (Casy I cell counter + analyzer system model SCAREF System GmbH).

**Statistical analysis:** All data are expressed as mean±SEM. Comparisons between two groups were performed using an unpaired Student's t-test. Differences were considered significant when p<0.05.

**RESULTS**

**Characterisation of the chemoattraction response of NK92 cells to recombinant human IP-10:** In this study, we have used the NK92 cell line that has been generated by Dr. Howard Young (National Cancer Institute, Frederick USA) to determine if medium conditioned by hepatocyte culture can exhibit a functional chemoattractant effect on immune cells. To-standardise chemotaxis, NK92 cells have been used for cell migration and assessed how this was influenced by a standard chemokine (recombinant human IP-10). As shown in Fig. 1 and Table 1, human NK92 cells were attracted to recombinant human IP-10 in a concentration-dependent manner. Comparison of the number of cells that migrated toward IP-10 showed an increase with time of incubation and amount of human IP-10. Comparison of passed cells showed that 40 and 100 ng mL⁻¹ IP-10 at 3, 6 and 24 h produced a significant increase in passed cells when compared to control. However, IP-10 at 10 ng mL⁻¹ produced no significant effect. Comparison of membrane-attached cells showed that IP-10 (40 and 100 ng mL⁻¹)

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**Fig. 1:** Chemoatraction response of human NK92 cells to recombinant human IP-10 points. NK92 cell migration was assessed with transwell microchemotaxis chambers. Recombinant human IP-10 (10, 40 and 100 ng mL⁻¹) in RPMI1640 supplemented with 10% (v/v) foetal calf serum and antibiotics was used to verify the chemoattractive response of the NK92 human cell line and this was placed in the lower compartment of the chamber. Cells (0.5×10⁵ mL⁻¹) were suspended in RPMI1640 supplemented with foetal calf serum 10% (v/v) and antibiotics and then were placed in the upper compartment of the wells. A 6.5 μm pore size polycarbonate filter, coated with fibronectin, separated the two compartments. After incubation (for 3 h) at 37°C, the filter was removed, stained with Jenner-Giemsa and cells that had migrated across the membrane were counted. Figure 1 shows a representative picture of Jenner-Giemsa stained cells that had migrated across into the membrane. RPMI1640 supplemented with foetal calf serum was used as control. Control was RPMI1640 medium supplemented with 10% (v/v) foetal calf serum.
Fig. 2: Chemoattractive response of human NK92 cells to medium from primary hepatocyte cultures. NK-92 cell migration was assessed with transwell microchambers chambers. Samples obtained from the medium from primary hepatocytes, cultured in basal culture, was placed in the lower compartment of chambers. NK92 cells (0.5×10^6/mL) suspended in RPMI1640 supplemented with 10% fetal calf serum and antibiotics was placed onto the wells in the upper compartment. A 6.5 µm pore size polycarbonate filter, coated with fibronectin, separated the two compartments. After incubation for 3 h at 37°C, the filter was removed, stained with Jenner-Giemsa and the cells that had migrated across the membrane were counted. Fig. 2 shows a representative picture of Jenner-Giemsa stained of the cells migrated across into the membrane. Incubation medium was used as control.

<table>
<thead>
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<th>Condition (ng mL⁻¹)</th>
<th>h</th>
<th>Passed</th>
<th>Membrane attached</th>
<th>Total attached</th>
<th>Membrane attached cells</th>
<th>Total attached cells</th>
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<tbody>
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<td>3</td>
<td>2532.8±385</td>
<td>1796.9±34</td>
<td>4329.8±339</td>
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<td>1846.9±30</td>
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<td>4877.1±542</td>
<td>3323.0±498</td>
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<tr>
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<td>2283.0±108</td>
<td>8248.8±565*</td>
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<td>3233.5±352*</td>
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<tr>
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<td>145886.0±12177</td>
<td>3916.0±27</td>
</tr>
</tbody>
</table>

Table 1: Chemoattractant response of human NK92 cells to different concentrations of recombinant human IP-10

Table 2: Chemoattractant response of NK92 cells to supernatant medium of primary rat hepatocytes at different time points

NK92 cell migration was assessed with transwell microchambers chambers. After incubation of NK92 cells with different dilution of recombinant IP-10 for specific time at 37°C, the filter was removed, stained with Jenner-Giemsa and cells migrated across the membrane were counted. Results are expressed as means±SEM for 4 independent experiments. (*p<0.05 v control at each time point (RPMI1640 supplemented with fetal calf serum was used as control)).

produced increased numbers of attached cells (except for 40 ng mL⁻¹ at 3 h). Again there was no effect with 10 ng IP-10 mL⁻¹. Comparison of the total number of attached cells showed, again, significant stimulation at 3, 6 and 24 h with 40 ng mL⁻¹ and 100 ng mL⁻¹ IP-10.

Analysis of the chemotaxis response of NK cells to medium from primary hepatocyte cultures: Following verification of the chemotaxis of NK92 cells in response to recombinant human IP-10, NK92 cells were incubated with medium samples obtained from primary hepatocyte cultures. As described in Fig. 2 and Table 2, NK92 cells exhibited a chemotactic response to medium harvested from primary hepatocyte cultures. Immediately after isolation (0 h), there are factors in hepatocyte medium that produce a significant attraction of NK92 cells. There was also a chemotactic activity with medium that had bathed hepatocyte cultures for 3 h and this activity was about twice that in the medium immediately after isolation. The two-fold change in chemotactic activity was found even after the cell suspension has been diluted (by mixing 1 mL cell
suspension and 1 mL fresh medium) for the first 3 h of culture (the inoculation period). After replacement of medium at 3 h with fresh maintenance medium, analysis of chemotactic activity at 24 h of culture showed that significant continued production of chemotactic factors had occurred.

**DISCUSSION**

Isolated and cultured hepatocytes express several different chemokines (Wang et al., 1999; Hassanshahi et al., 2007a). Although we identified that medium from hepatocyte cultures contains specific chemokines by immunoblotting (Varley et al., 2003; Hodge et al., 2002; Hassanshahi et al., 2007b), there is potential that migration assays detected yet other chemokines and other factors such as complement components. In this report, we demonstrated that hepatocytes expressed factors that were chemoattractive for human NK92 and peripheral blood leukocytes and that the factors must interact with the repertoire of receptors responsible for recruitment of these cells. Preliminary results showed that human NK92 cells migrated in response to recombinant human IP-10 and to hepatocyte culture medium isolated at different time points of culture. Expression of CXC chemokine receptors has been reported for NK cells (Hodge et al., 2002; Koniaris et al., 2001). NK92 cells migrated in response to hepatocyte culture medium recovered from freshly isolated cells. As we shown in our other reports, at that time point IP-10/Mob-1 expression was low (by immunoblotting) (Hodge et al., 2002; Hassanshahiraviz et al., 2006; Salkowski et al., 1998) but immunoblotting identified that SDF-1α and Gro/KC expression was high (Hodge et al., 2002; Hassanshahiraviz et al., 2006). Constitutive expression of SDF-1α and Gro/KC from hepatocytes immediately after isolation may account for the migration of NK92 cells. The migration activity was increased with medium taken after 3 h of culture and this could reflect production of other chemokines, such as IP-10/Mob-1 (confirmed by immunoblotting) and other chemotactic factors. The amount of chemotactic factor(s) decreased between 3 and 24 h of culture and this paralleled the decreased expression of certain chemokines when detected by immunoblotting (Varley et al., 2003; Hodge et al., 2002; Hassanshahiraviz et al., 2006). Although there have been no reports of attraction of NK92 cells toward culture medium from hepatocytes, there have been studies in vivo to show attraction of NK and activated T cells to sites of liver injuries (Shields et al., 1999). Treatment of mice with IL-2 and/or IL-12 caused rapid recruitment of T and NK cells to the liver in an IP-10/Mob-1-dependent pathway (Park et al., 2001) and mice hepatocyte culture supernatants caused migration of human and mouse T cells and NK cells.

In contrast to results seen for NK92 cells migration of peripheral blood leukocytes was slightly less in response to medium from 3 h of culture than that from freshly isolated hepatocytes. It should be noted that almost all peripheral blood leukocytes underwent migration and the sensitivity of the functional migration assay may be limited by conditions of the assay. As mentioned earlier, a population of cells sized between 9 and 14 μm (around the size of NK cells) passed the membrane pores and it has been shown that the NK cells express CXCR1, CXCR2 and CXCR3 and CXCR4. Therefore, this 9-14 μm population can be viewed as positive cells for CXCR3 and CXCR4. Over all this results showed that the medium from cultured hepatocytes is a source of factors involved in recruitment of sub-populations of leukocytes especially NK cells. In agreement with these results, other studies in vitro have also shown that recombinant human IP-10 and rat Mob-1 attracted lymphocytes and monocytes (Taub et al., 1995).

**REFERENCES**


