ELSEVIER

Journal of Hepatology 45 (2006) 60–71

Journal of Hepatology

www.elsevier.com/locate/jhep

Anti-fibrotic activity of NK cells in experimental liver injury through killing of activated HSC

Alaa Melhem[1,†], Nidal Muhanna[1,†], Amal Bishara[2], Carlos E. Alvarez[3], Yaron Ilan[1], Taiser Bishara[1], Amjad Horani[1], Mithal Nassar[1], Scott L. Friedman[3], Rifaat Safadi[1,*]

[1]*Division of Medicine, Liver and Gastroenterology Units, Hadassah University Hospital, Pob 12000, 91120 Jerusalem, Israel*
[2]*Tissue Typing Unit, Hadassah University Hospital, Jerusalem, Israel*
[3]*Division of Liver Diseases, the Mount Sinai School of Medicine. New York, NY 10029, USA*

***Background/Aims*: We have investigated the role of natural killer (NK) cells in hepatic fibrogenesis. Mouse NK cells express both inhibitory/activating-killing-immunoglobulin-related-receptors (*iKIR/aKIR*) specific for Class-I-molecules.**

***Methods*: Hepatic fibrosis induced by carbon-tetrachloride (CCl_4) was compared between wild-type (WT) male-BALBc; combined-immunodeficiency (SCID, lacking B/T-cells); and SCID-BEIGE-mice (lacking B/T/NK cells), and naive mice.**

***Results*: Hepatic fibrosis significantly increased in all CCl_4-treated groups. SCID-BEIGE mice had more fibrosis than SCID-mice ($P < 0.0001$) as assessed by morphometry of sirius-red stained tissue sections. Following fibrosis, hepatic NK cells significantly decreased, the *aKIR:iKIR*-ratio significantly increased while Class-I expression on HSC decreased ($P < 0.001$). Both freshly isolated and in situ HSC displayed a significant increase in cellular apoptosis following fibrosis induction. Confocal microscopy demonstrated the direct adhesion of NK cells to HSC in mouse liver sections and in vitro human NK/HSC co-culture. In human HSC there was decreased Class-I expression and increased apoptosis as well, which was further increased following blocking of either HSC-related Class-I or NK-related killer inhibitory receptors. Apoptosis was inhibited by pre-incubation of NK cells with the granzyme inhibitor 3,4-dichloroisocoumarin.**

***Conclusions*: During liver injury, NK cells have an anti-fibrotic activity at least in part through stimulation of HSC killing.**

***Keywords*: Hepatic stellate cells; Hepatic fibrosis; NK cells; Lymphocytes; SCID; KIR**

1. Introduction

The cellular basis of hepatic fibrosis involves the interplay of many factors and cells including hepatic stellate cells (HSC), hepatocytes, Kupffer cells, endothelial cells, platelets and lymphocytes [1]. In liver injury, Th2 lymphocytes, characterized by elevated IL-4, appear to promote fibrogenesis relative to Th1 lymphocytes that express high levels of interferon (IFN) gamma [2–10]. In our earlier studies, transgenic expression of hepatic IL-10 led to reduced fibrosis and significant alterations in lymphocyte subsets following the induction of fibrosis [11]. Using a model of adoptive transfer for hepatic fibrosis, hepatic fibrosis was associated with a significant decrease of CD4 subsets, whereas cytotoxic CD8 subsets harboured fibrogenic potential, and the anti-fibrotic response of IL-10 was associated altered CD4/CD8 ratios in liver.

Natural killer (NK) cells are a key component of the innate immune system and play a critical role in the early stages of the immune response against tumour cells, as well as those infected by viral and microbial pathogens. They recognize host cells through the interaction of receptors on the NK cell surface with MHC Class-I molecules expressed by infected antigen-presenting cells

Received 24 August 2005; received in revised form 30 November 2005; accepted 15 December 2005; available online 8 February 2006

* Corresponding author. Tel.: +972 2 6777337; fax: +972 2 6420338.
E-mail address: safadi@hadassah.org.il (R. Safadi).
† Both authors contributed equally.

0168-8278/$32.00
doi:10.1016/j.jhep.2005.12.025

(APC) [12]. The activated NK cells then produce lymphokines, including interferons, to recruit additional cells to the site of inflammation.

Activated NK cells also become cytolytic, effecting the destruction of the tumour or infected target cells. NK cells express receptors capable of binding classical Class-Ia MHC proteins and regulating NK-cell functions through a number of cell surface receptors. Specifically, a system of recognition in human tissues is mediated by killer immunoglobulin-related receptors (*KIR*) on these cells [13]. In mice, Ly-49 proteins, which are type II transmembrane proteins of the C-type lectin superfamily, carry out *KIR* activities [14,15]. The human *KIR* and Ly-49 receptors are capable of distinguishing individual classical Class-I MHC alleles [16,17]. Despite major structural differences, Ly-49 and human *KIR* receptor families are functionally equivalent. The best characterized members of the Ly-49 family are the *inhibitory KIR* (*iKIR*) that includes Ly-49A, C, and G [16–25]. Ly-49 inhibitory receptors engage Class-I MHC molecules on normal cells and negatively regulate NK function, leading to inhibition of killing by the NK cell [16,23,26,27]. This type of inhibition is lacking when tumour cells or virally infected cells that have down-regulated Class-I MHC expression are encountered, and NK cells become activated [28]. In contrast, engagement of an *activating* Ly-49 receptor (*aKIR*), for example LY-49D, initiates a cascade of signalling events leading to NK activation enabling the destruction of the tumour or infected target [24,25,29–35]. It appears that during NK contact with healthy cells, inhibitory receptor signals are dominant over activating receptor signals, preventing auto-attack and thus maintaining NK self tolerance [14,36].

In this study, we explored the possibility that NK cells are immune modulators of activated HSC and are subsequently associated with either a pro- or anti- fibrogenic effect. Accordingly, we evaluated the role of the NK cells in hepatic fibrosis in immunodeficient animal models in order to address this question.

2. Materials and methods

2.1. Animals

Ten-week-old BALBc and C57BL/6 male mice were purchased from Taconic and Harlan Laboratories respectively. BALBc severe combined immune deficient (SCID) mice were housed in a barrier facility. Animals received care according to National Institutes of Health guidelines.

2.2. Induction of hepatic fibrosis

Hepatic fibrosis was induced using carbon tetrachloride (CCl_4). CCl_4 was diluted to 10% in corn oil and introduced by intra-peritoneal injections at dosages of 0.5 μl pure CCl_4/g body weight twice a week, for 4 weeks. Following Ketamine/Xylazine anaesthesia, animals were sacrificed, and serum, livers and cells were harvested 3 days after the final dose of CCl_4.

2.3. Animal groups and experimental design

To evaluate the role of NK cells in HSC activation, hepatic fibrosis was induced in 3 groups of BALB/c mice (*eight animals were included in each animal group*): wild type (WT), SCID mice (genetically lacking B and T lymphocytes), SCID BEIGE (lacking B, T and NK cells), and were compared to untreated naïve groups. The BALB/c background was selected in this part of experiment due to the commercially availability of SCID BEIGE mice. The doubly mutant SCID BEIGE mice have reduced NK cell activity, in comparison to SCID mice [37], making them suitable to investigate the role of NK cells [38,39].

To investigate the NK stimulation and its *aKIR*/*iKIR* alterations following fibrosis, the mRNA expression of LY49A2-(*i*KIR) and LY49L1-(*a*KIR) were tested on splenocytes from naïve and fibrotic WT BALBc mice. As those receptors are mainly expressed over NK cells, the results are reflecting their status in NK cells.

The same fibrosis model was also induced in WT C57BL/6 mice and compared to its naïve group (eight animals were included in each animal group). C57BL/6 rodents were used in these experiments since specific fluorescent antibodies for *anti-iKIR* and *anti-aKIR* staining are commercially available. However, no FACS antibodies are available recognizing BALB/c.

2.4. Tissue RNA extraction and aKIR/iKIR mRNA detection

Total cellular RNA was extracted from freshly isolated splenocytes from naïve and fibrotic WT BALBc mice using Trizol reagent® (GIBCO-BRL, Life Technologies) and DNA digestion. RNA was then used as a template for reverse transcription into single strand cDNA using the Reverse Transcriptase System (Promega Corporation, Madison, WI). Synthesized β-Actin, LY49A2-(*i*KIR) and LY49L1-(*a*KIR) cDNA were detected by semi-quantitative RT-PCR with the following primers: *β-Actin* (*as a housekeeping gene*)—*Forward*: 5′gat gag att ggc atg gct tt 3′, *β-Actin-Reverse*: 5′aga gaa gtg ggg tgg ctt tt3′. *LY49A2-(iKIR)—Forward*: 5′cca caa taa ctg cag caa cat gc 3′, *LY49A2-(iKIR)—Reverse*: 5′ ggt caa aac act tgt ttg caa gg 3prime;. *LY49L1-(aKIR)—Forward*: 5′gct cat tgt gat agc tct cgg 3′, *LY49L1-(aKIR)—Reverse*: 5′gct taa gga act tca gtt cat cc 3′.

2.5. Real-time PCR analysis

Liver RNA was extracted as described above. Synthesis of cDNA was performed using 2 μg of total RNA per sample with random primers and reagents contained in the Reverse Transcription System kit, according to the manufacturer's protocol (Promega Corporation, Madison, WI). The reverse transcriptase product was diluted 20 × in nuclease-free H_2O and 5 μl of each sample was loaded into 96 well plates for real-time PCR in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). β-Actin and α2-macroglobulin served as internal controls and H_2O served as a negative control. Amplification reactions included oligonucleotide primers for each target gene, and for β-actin and α2-macroglobulin, as well as platinum Taq polymerase and SYBR Green DNA-binding dye. Fluorescence signals were analyzed during each of 40 cycles (denaturation 15 s at 95 °C, annealing 15 s at 56 °C and extension 40 s at 72 °C). [Denaturation curves of target genes and ß-actin, performed at the end of the PCR], and detection of the PCR products by agarose gel electrophoresis confirmed the homogeneity of the DNA products. Relative quantification was calculated using the comparative threshold cycle (C_T) method [as described in the User Bulletin #2, ABI PRISM 7700 Sequence Detection System]. C_T indicates the fractional cycle number at which the amount of amplified target genes a fixed threshold within the linear phase of gene amplification, and is inversely related to the abundance of mRNA transcripts in the initial sample. Mean C_T of duplicate measurements would be used to calculate ΔC_T as the difference in C_T for target and reference. ΔC_T for each sample was compared to the corresponding control C_T and expressed as ΔC_T. Relative quantity of product was expressed as fold-induction or repression of the target gene compared to the control primers, according to the formula $2^{-\Delta C_T}$. Primers used for the IL-4 were *Forward*: ATG GGT CTC AAC CCC CAG CTA