RESEARCH COMMUNICATION

Increased Expression of IL-6 mRNA in Hepatocellular Carcinoma Cell Lines Correlates with Biological Characteristics

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Abstract

Background & aims: IL-6 has been implicated in both virus-associated and diethylnitrosamine-induced hepatocellular carcinomas (HCCs). Generally it is produced by immune cells such as Kupffer cells inthe liver. To understand mechanisms by which IL-6 might participate in the genesis of HCCs, the production of IL-6 by cell lines under different conditions was examined to determine inducing factors Methods: Expression of IL-6 mRNA in both hepatoma cell lines and a normal liver cell line L-02 was measured by quantitative RT-PCR. Biological molecules including liposome, dsRNA and cell debris were used to stimulate IL-6 mRNA expression in HepG2 cells and inhibition was effected by RNAi. Proliferation was assessed by MTT and clone formation and migration was determined by scratch assay. Results: All of the HCC cell lines observed expressed IL-6 mRNA, including HepG2, Bel-7402(7402), MHCC-97H and SMMC-7721.Normal liver cell line L-02 also expressed IL-6 mRNA. SiRNA to IL-6 specifically knockdowned IL-6 mRNA expression in HepG2, and liposome, dsRNA and cell debris increased it. Both proliferation and migration of HepG2 cells were related to the level of IL-6 HepG2 expressed. Conclusion: Both normal liver cell line and HCC cell lines can produce IL-6 so that Kupffer cells are noit the only source of the cytokine in the liver well as other immune cells. That the fact that HCC cells reacted to stimulation of biological molecules such as liposome, dsRNA or cell debris with increasing production of IL-6 indicates that the cytokine might play an important role not only in the period of tumor initiation but progression and recurrence as well.

Keywords: HCC cells - IL-6 - debris - DAMPs - dsRNA

Asian Pacific J Cancer Prev, **12**, 3361-3365

Introduction

The implication of inflammatory mechanisms in the development of malignancies has been suggested for long since the nineteenth century by the Prussian scientist Rudolph Virchow (Balkwill et al., 2001) and received further attention in recent years (Engels, 2008). Hepatocellular carcinoma(HCC), the most common primary liver cancer (Ferlay et al, 2010), is one of the examples (Berasainet al., 2009). HCC is a dreaded complication of chronic liver disease that occurs in the setting of risk factors including hepatitis B (HBV) and hepatitis C (HCV) viral infections, alcoholic liver disease, hemochromatosis, and nonalcoholic steatohepatitis. Even though the different etiology, most HCC appears in cirrhotic livers after years of chronic inflammation. Growing evidences indicates that, besides the other causes, inflammation plays an important role in the initiation and progression of HCC (El-Serag et al., 2007; Michielsen et al., 2011; Pinzani et al., 2011; Weber et al., 2011). Pro-inflammatory cytokines, generated by macrophages, Kupffer cells in liver, or lymphocytes in the inflammatory tumor microenvironment, serve as innate immune mediators leading tumor cells to exert anti-apoptotic and pro-angiogenic effects (Sato et al., 2009). Among these mediators, interleukin-6 (IL-6) has been shown to play a key role in HCC initiation and development (Allavena et al., 2007).

Epidemiological studies have confirmed the association between the inflammatory conditions of the liver correlate with high circulating IL-6 levels, which are even more elevated in patients who develop HCC (Naugler & Karin., 2007). Observations made in genetic experimental models of cancer have also suggested that IL-6 trigger hepatocyte proliferation, liver regeneration, and animal survival after partial hepatectomy (Abshagen et al., 2007). Recently, it has been reported that, in a chronic inflammation and cancer mouse model induced by diethylnitrosamine (DEN), high IL-6 level in male mice is consistent with the high incidence of HCC development. By inhibition of IL-6 production, estrogen reduces liver cancer risk thereby explaining the gender differences in hepatocarcinogenesis (Naugler et al., 2007).

It has been suggested that macromolecules released

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during hepatocyte necrosis act as ligands to Kupffer cells and trigger TLR–MyD88 signaling and IL-6 synthesis, which promote compensatory hepatocyte proliferation (Maeda et al., 2005). In addition, at the hepatocyte level, parenchymal cells express high amounts of IL-6 receptor (Gao, 2005). All these data suggest that IL-6, generated in the hepatic microenvironment, is intimately linked to the development of hepatoma.

Besides Kupffer cells, the studies in our laboratory, togather with other researchers, have shown the liver cells as well as HCC cells could produce IL-6. Xiang et al. (2011) reported recently that HCC cells synthezize IL-6 induced by HBx. In this study, we report that HCC cells produce IL-6 induced by the cell debris, dsRNA, and liposomes (LP). These indicate that there be another mechanism of IL-6 production.

Materials and Methods

Cell lines and culture conditions

Human HCC cell line HepG2 and Bel-7402 (7402) were presents generally from professor Guanxin Shen, Dept of immunology, Tongji medical school, Huazhong Science University and MHCC-97H (97H) was from Zhongsan Hospital (Shanghai, China). Normal liver cell line L-02 and HCC cell line SMMC-7721 (7721) was provided by China center for type culture collection (CCTCC). All cells were cultured as described (Yuan FJ et al., 2008). Cell debris was prepared from the cultured cells by repeated freeze-and-thaw treatment from -80°C to 37°C. Liposome and dsRNA treatment was as same as RNAi assay.

RT-PCR and real-time RT-PCR

The cultured cells were collected before use. Primers used (Table1) for each gene were designed based on the Genebank sequence and were introns panning and were synthesized by Sangon (Shanghai, China). Total RNA extraction of the cultured cell lysates with Trizol reagent (invitrogen). Extracted RNA was dissolved in diethylpyrocarbonate-treated water (DEPC water). cDNA was synthesized with the RevertTMAid first strand cDNA synthesis kit (Fermentas, Canada). PCR was performed with PCR MasterMix (Tiangenin Biotech, Bejing) of 20 μl volume, and if processing Real-time PCR, 0.4 μl of 20×SYBR Green (Sigma)was added. For each PCR, an aliquot of cDNA (diluted 1:20) was used. Real-time PCR was performed on a Rotor-gene 6000 SYSTEM (Corbett Research, Mortlake, New south Wales, Australia) according to the program formulated in pre-tests. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the normalization control in both Real-time RT-PCR and normal RT-PCR. The presence of a single, right-sized PCR product was confirmed by running the samples on 2% agarose gels.

Determination of cell proliferation by MTT assay. After the various treatments, aliquots of 100 μl of suspension were distributed in 96-well culture microplates at a density of 1×10^6 cells/ml. 10 μl of 3 - (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium blue (MTT, Sigma) was added at a concentration of 5 mg/ml in well. Each sample was analyzed in hexa-plicate to control variation. Cells were incubated at 37°C in 5% CO2 for 4 h and then the medium was discarded and 150 μl of dimethyl sulfoxide (Me2SO) was added to each well. The optical absorbance was read at 570 nm using microplate reader (BioRad 550, USA). The cell survival ratio (CSR) was calculated according to the formula: CSR=A570 experiment/A570 control ×100%.

RNAi assay

The SMART pool small interfering RNA (siRNA) for human IL-6 was purchased from Dharmacon Research (Shanghai genePharma Co., Ltd, Shanghai, China) and transfected into HepG2 cells using LipofectAMINE 2000 reagent (Life Technologies, Inc., Gaithersburg, MD) . Cells were extracted 24 h after transfection, and the mRNA levels of IL-6 and other designed genes in cell lysates were analyzed by Real time-PCR.

Clone formation in soft agar

Briefly, single-cell suspensions were prepared with trypsin and then agarose was mixed in a final concentration of 0.3%. Aliquots of 1.5ml containing 100 cells and 10% fetal bovine serum (Hyclone, Logan, USA) were plated in triplicate on 35-mm culture dishes (Nunc,Roskilde, Denmark) over a base layer of 0.6% agarose (Biowest, Miami, USA) and allowed to gel. Colonies of >50 cells were counted after 14 days of incubation.

Scratch assay

Scratch wound was created in a confluent monolayer of HepG2 cells with a 10 μl tip, washed twice with PBS and the medium supplemented with 2% FCS was replaced (GibcoBRL). Images of the wound area were taken at 0, 24 and 48 h post-scratching. The width of the wound was measured using Image-pro Express and migration rate (MR) = (space0-spacem)/ space0×100%, here the space0 means the scratch space at beginning, spacem, the scratch space at measuring.

Statistical analyses

All tests were repeated at least three times. Data are represented as the mean ± SEM and were analyzed for statistical significance by Student's test

Results

IL -6 and MyD88 mRNA was expressed in both L-02 liver cell line and HCC cell lines.

While we examining expression of IL-6 and MyD88 in HCC cell lines with RT-PCR, we found out that it expressed in all of the HCC cell lines we studied including HepG2, Bel-7402, SMMC-7721 and 97H (Figure 1). Besides, it also expressed in normal hepatocell line L-02. The primers used in PCR were demonstrated in table 1.

Knockdown of IL-6 inhibited the proliferation and migration of HepG2 cells.

To verify that IL-6 produced by HCC cells play a role in HCC development, we investigated the implication of IL-6 in the cell biological features after it was

Table 1. Sequences of the Primers in PCR

Gene name	Primer	Sequence	Product
IL-6	Forward	5'-TCGAGCCCACCGGGAACGAA-3'	
	Backward	5'-GTGGCTGTCTGTGTGGGGCG-3'	193
MyD88	Forward	5'-GCACATGGGCACATACAGAC-3'	
	Backward	5-'TGGGTCCTTTCCAGAGTTTG-3'	239
GAPDH	Forward	5'-CTTTGACGCTGGGGCTGGCA-3'	
	Backward	5'-TGGCAGGGACTCCCCAGCAG-3'	257

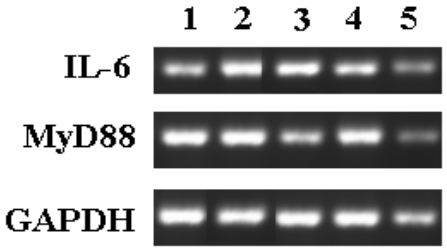

Figure 1. Both Normal Liver and HCC Cell Lines Express IL-6 as Well as MyD88 mRNA. 1.L-02; 2. Bel-7402; 3.HepG2; 4. MHCC-97H; 5. SMMC-7721

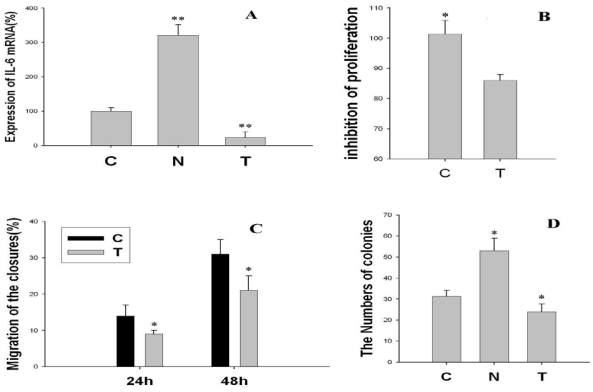

Figure 2. Knockdown of IL-6 Inhibited the Proliferation and Migration of HepG2 Cells. C: control, the cells received no treatment; N: transfected with non-specific dsARNA; T: transfected with SiRNA to IL-6. *: p≤0.05; **: p≤0.01. A. Transfect of SiRNA to IL-6 knockdowned expression of IL-6mRNA to 20% of the control(n=3). B. Knockdown of IL-6 inhibited the proliferation of HepG2 to 86% of the control(n=6); C. Cell migration slowed down after RNAi to IL-6, at 24 h, or 48 h(n=3); D. Clone formation decreased from 31.33±2.87 in control to 24±3.74 in tested cells. Especially, transfect of non-specific dsRNA induced clone formation to 53±5.96 (n=3)

knockdowned by RNAi. 4 pieces of SiRNA oligos were tested and one with best repressive effect was used in following experiments. The sequence of the SiRNA oligo is as follows: 5'-CUUCCAAUCUGGAUUCAAUTT-3'. 24 h after the transfect of the SiRNA to IL-6 into the HepG2 cells, the cells were collected and q-RT-PCR was performed. The results showed (Figure 2A) that IL-6 was knockdowned efficiently to 20% of the original HepG2 cells (control,C). On the basis of this, some other tests were performed: Firstly, while measuring the proliferation by MTT, the test showed that the HepG2 cells prolifered slower after IL-6 was inhibited by RNAi than before, though slightly, but with significant difference statistically (Figure 2B). RNAi to IL-6 repressed the migration of

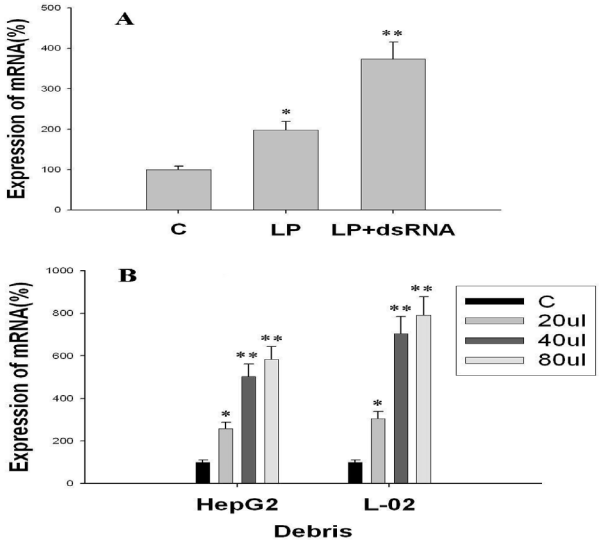

Figure 3. Expression of IL-6 mRNA Increased in HepG2 when Exposure to DAMPs. *p≤0.05; **p≤0.01. A. HepG2 cells exposed to liposome(LP) or dsRNA oligo(LP+dsRNA) lead to higher expression of IL-6 mRNA than control(C), and the latter was much higher 2 times than the former(n=3). B. The level of IL-6 mRNA was consistent with amount of the cell debris added into the culture. While the quantity of debris doubled from 20ul to 40ul, the level of mRNA doubled equally

HepG2 too. 24 h after the SiRNA was transfected into the cells, the scratch test began. After another 24 h, the cells move out about 9±1% of the original space vs. 14±3% of the control cells; 48 h later, the tested cells move out 21±4% and the control cells, 31±4%. The migration reduced about 30-40% from the control HepG2 to the IL-6 knockdowned cells (Figure 2C). Clone formation assay was also performed and the numbers of colonies of HepG2 reduced from 53±5.96 in the non-specific controle (NC), namely the cells transfected with non-specific dsRNA, to 24±3.74 (Figure 2D). The reduction of colonies was coincident with the decrease of the IL-6. It was attentioned that the clone number of the control cells which was not transfected with anything, even not contact with liposome, was only 31.33±2.87, fewer than the NC.

Increased expression of IL-6 mRNA induced by DAMPs in HepG2.

To understand how IL-6 expressed in HCC cells, we chose HepG2, the Hepatoma cell line, for more observation on its reaction to the stimulation coming from the biological molecules including liposomes (LP), liposome+dsRNA (LP+dsRNA), and cell debris.

Liposome and dsRNA. Firstly in our RNAi test, we found that IL-6 mRNA expressed higher in the cells received the LP control than the cells received nothing,

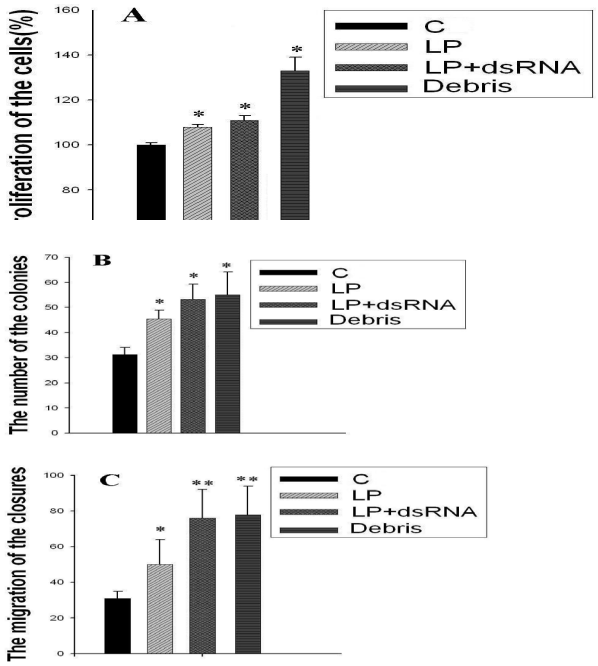

Figure 4. Exposure to DAMPs Improved the Proliferation and Migration of HepG2 Cells. *p≤0.05; **p≤0.01. A. Cell proliferation was measured by MTT. The cells grew faster after co-culturing with LP or dsRNA and fastest with cell debris for 24 h(n=6). B. The clone numbers increased respectively to different biological molecules after exposure for 24 h to LP, dsRNA or cell debris. LP increase the clone numbers the least and cell debris, the highest. C. All of the three DAMPs prompted the migration of HepG2 and same as the former two tests, the potence of the three was successively LP<LP+dsRNA<cell debris

and it expressed even much higher when the cells received stimulation from mix of the non-specific dsRNA mimics and LP. Repeating the tests, the phenomenon always happened. Then we doubled the quantity of LP or LP+dsRNA, consistently, the expression of IL-6 mRNA was increased (Figure 3A). Examination on the cDNA of other tests, RNAi to OCT4 test we did before (Yuan FJ et al., 2010), came to similar results. All of the IL-6 mRNA expressions in cDNA from the cells treated with LP, LP+non-specific dsRNA, or dsRNA to OCT4, were higher than that of without any treatment. We recognized that it was the liposome and dsRNA that increased the expression of IL-6 mRNA (Data not shown).

Some authors reported that lipopolysaccharides (LPS) exposure could induce the expression in HepG2 (Gutierrez-Ruiz MC et al., 2001). If LP be contaminated by LPS, it could rise the IL-6 mRNA expression in HepG2. Disappointedly, the results were ambigous while HepG2 cells were exposed to LPS, even the concentration of LPS was up to 10 ug/ml (Data not shown). Therefor, LP elevated the IL-6 mRNA not for LPS logically.

Cell debris. Liposome is someway similar to membrane and dsRNA belongs to biological molecules. Both are produced during cellular stress or released by necrotic and apoptotic cells (Kawai & Akira, 2007), the so called damage-associated molecular patterns(DAMPs) (Matzinger P, 2007). Based on the analysis, we supposed that cell debris should induce the expression of IL-6 mRNA. Repeated freeze-and-thaw treatment on the cultured HepG2 to break the cells, and the HepG2 cells were co-cultured with the broken debris for 24h. Examination showed the expression of IL-6 mRNA increased more than 5 times in the HepG2 cells after co-cultured with the broken cells. Since Hepatitis B virus X protein stimulates IL-6 expression dramatically, and HepG2 expresses the HbsAg, further study of co-culture of HepG2 with L-02 cell debris was carried out to exclude the possibility of the stimulation by HBV molecules. Same as HepG2 debris, exposure to L-02 debris also resulted in vigorous expression of IL-6 mRNA (Figure 3b).

DAMPs prompted the proliferation and migration of HepG2.

It is interesting that HepG2 respond to the cell debris with increased IL-6 mRNA expression. Observation on thire roles played to HepG2 was followed. Exposure of HepG2 respectively to LP, LP+dsRNA and cell debris for 24h and cell proliferation as well as migration was determined with MTT (Figure 4A), clone formation (Figure 4B) and Scratch assay (Figure 4C). The results showed all of the three kinds of DAMPs prompted the proliferation as well as migration of HepG2. Especially, the increase both proliferation and migration were respectively corresponding to the level of IL-6 elevated (Figure 3).

Discussion

Carcinogenesis, consisting of initiation, promotion and progression, is a multistage process governed by cumulative genetic and epigenetic alterations (He et al., 2010). Associated to the alterations, microenvironment and inflammation may be a frequent tumor promoter in each period. Growing studies have indicated that IL-6 is involved deeply in HCC development (Berasain et al., 2009). This study showed that both L-02, a so called normal liver cell line, and some HCC cell lines including HepG2 produce IL-6, a phenomenon was also found by others (Xiang et al., 2011). In the process of DEN-induced hepatocarcinogenesis, IL-6 was demonstrated playing a role in initiation of HCC. The fact that L-02 produce IL-6 lead us to suppose that hepatocytes do same as immune cells. So the role of IL-6 in HCC development might be more than what we thought before. IL-6 is expressed in HepG2, indicated it play a role in the HCC progressing too. Besides, recently Lee TK et al have demonstrated that CD24+ phenotype tumor stem cells (Tumor-initiating cells) in liver drive self-renewal and tumor initiation through STAT3-mediated Nanog regulation (Berasain et al., 2009). If IL-6 be expressed in tumor cells, it might activate STAT3 through IL-6-STAT3 pathway and take part in the recurrence of HCC. RNAi to IL-6 lower the ability of proliferation and metastasis of the HepG2 surport the opinion too. Still, these need more study.

Ordinarily, immune cells respond to cell debris with IL-6 production (Lee et al., 2011). Now our observation has shown that HepG2 cells, like immune cells, increase their expression of IL-6 while responded to cell debris. Carefull analysis of the expression of IL-6 between the

HepG2 cells treated with liposome, the non-spicific dsRNA oligo and that with nothing, we determined that HepG2 cells also responded to the liposome and liposome-RNA oligo mix and elevated their IL-6 expression. As to our knowlege, this is the first report of this kind. Hepatoma cells respond to DAMPs with increased IL-6 expression intimate another mechanism by which IL-6 play a role in the HCC of non-infectious etiology. The observation that the DAMPs stimulation boosts the proliferation and metastasis of HepG2 might be a support for the suggestion. The augment in proliferation is in some way different from the compensatory hepatocyte proliferation (Kono et al., 2008) after ablation of IKKb in hepatocyte, because of enhanced reactive oxygen species (ROS) production, increased JNK activation, and hepatocyte death.

It has been found that HCC cells express TLR2, TLR3, TLR4, TLR6, TLR9, and each receptor recognize the corresponding ligands(Mencin et al., 2009). According to this, we suppose that other DAMPs such as intracellular molecules DNA, HSPs and High Mobility Group 1 protein (HMGB1) and extracellular molecules Hyaluronic acid, Heparan sulfate would be recognized by HCC cells. Whether HepG2 cells react to these molecules with elevated IL-6 production is now under tightly investigation. Since MyD88 play a critical role in induction of IL-6 by HBx, We are also looking for the posibility of MyD88 mediating the signal by which the DAMPs induce IL-6.

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