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The role of the IL-8 signaling pathway in the infiltration of granulocytes into the livers of patients with alcoholic hepatitis

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ABSTRACT

Background and aim: IL-8 (C-X-L motif chemokine ligase 8) and CXCR2 (C-X-C-motif chemokine receptor 2) are up regulated in alcoholic hepatitis (AH) liver biopsies. One of the consequences is the attraction and chemotactic neutrophilic infiltrate seen at the AH stage of alcoholic liver disease.

Materials and methods: Human formalin-fixed, paraffin-embedded (FFPE) liver biopsies from patients who have AH were studied by (2.1) RNA sequencing, (2.2) PCR and (2.3) semi quantitation of specific proteins in biopsy sections using immunohistochemical measurements of antibody fluorescent intensity with morphometric technology.

Results: Immunohistochemistry of IL-8 showed that the expression was increased in the cytoplasm of the hepatocytes in AH liver biopsies compared to the controls. IL-8 and ubiquitin were co-localized in the MDBs. Numerous neutrophils were found throughout and satellitosis of neutrophils around MDBs was present. This suggested that IL-8 may be involved in MDB pathogenesis. RNA seq analysis revealed activation by IL-8 which included neutrophil chemotaxis by LIM domain kinase 2 (LIMK2) (17.5 fold increase) and G protein subunit alpha 15 (GNA15) (27.8 fold increase).

Conclusions: The formation of MDBs by liver cells showed colocalization of ubiquitin and IL-8 in the MDBs. This suggested that IL-8 in these hepatocytes attracted the neutrophils to form satellitosis. This correlated with up regulation of the proteins downstream from the IL-8 pathways including LIMK2, GNG2 (guanine nucleotide binding proteins) and PIK3CB (phosphatidyl isitol-4, 5-biophosphate-3-kinase, catalytic subunit beta).

1. Introduction

CXCL8 (IL8) is a potent chemotactic factor for neutrophils (Masuhima et al., 1988). CXCL-8 is produced by a variety of cells including monocytes and epithelial cells and binds to the chemokine receptors CXCR1 and 2 (Lane et al., 2006; Rollins, 1997). Cb1 (120 KD protein tyrosine kinase binding domain) and AKT (RAC-alpha serine/threonine protein kinase) mediate the CXCL8-induced chemotactic pathway through the P13K and Cb1 complex pathway (Lane et al., 2006). Cb1 inhibits and AKT up regulates CXCL8-induced chemotaxis (Lane et al., 2006). The CXCR1/CXCR2 receptors mediate the CXCL8-induced chemotaxis (Feniger-Barish et al., 2002). LIMK2 is also involved in the recruitment of neutrophils to form the inflammatory infiltration (Mouneimne et al., 2006). The role of neutrophils in liver diseases (Xu et al., 2014) and in alcoholic hepatitis (AH) in particular, have been reviewed (Mookerjee et al., 2007; Jaeschke, 2002).

In a mouse model of alcoholic neutrophilic hepatitis the features of alcoholic hepatitis were induced, including the clinical features such as hypoalbuminemia, hyperbilirubinemia, and splenomegaly as well as neutrophilic liver infiltration (Lazao et al., 2015). More recently a mouse model that morphologically resembles alcoholic hepatitis (AH), including neutrophilic infiltrate, balloon cell change and Mallory-Denk body formation was reported (Wieser et al., 2016). What is so remarkable about this study is that pepducin's functional blockade of the CXCL-8 receptors 1 and 2 prevented the development of AH and also reversed AH after it had become manifest, including the neutrophilic infiltrate. The two receptors CXCR1/2 are critical for the recruitment and activation of neutrophils. Pepducin therapy also decreased the transcription of liver CXCL-8 in the liver. The authors proposed that high circulating CXCL-8 in human AH may cause pathogenic overzealous neutrophilic activation and that a therapeutic blockade with pepducin could prevent and reverse AH. Pepducin also abrogated pro-inflammatory cytokine transcription (IL-1B/CXCL-1, TNFα) and down regulated caspase 1 expression. In tissue culture studies of the Hep3b cell line and HepG2 cells both secreted CXCL-8 and attracted neutrophils in response to ethanol. This proved that hepatocytes are a

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source of CXCL8 when exposed to ethanol and that the response leads to neutrophil chemotaxis.

To determine changes in the expression of the IL-8 signaling pathway in liver biopsies from patients with AH, RNA sequencing was performed (Liu et al., 2015a). In this study many of the genes in the IL-8 signaling pathway were up regulated inducing LIMK2 (17.5 fold increase) and GNA15 (27.7 fold increase). In another study, using similar liver biopsies from AH patients, real time PCR analysis of CXCR2 further showed a 6 fold increase in expression compared to control normal livers. Likewise, IL-8 expression was up regulated 12 fold, supporting the concept that IL-8 was responsible for the neutrophil infiltration seen, as reported here (Liu et al., 2015b).

2. Materials and methods

2.1. Liver biopsy specimens

Human formalin-fixed paraffin-embedded (FFPE) liver biopsies from patients who had alcoholic hepatitis (AH: n = 8–10) were obtained from Harbor-UCLA hospital archives. In all the cases, liver forming MDBs were present. Normal control livers [n = 3] were used for comparison.

2.2. RNA isolation

RNA isolation of FFPE sections of human liver biopsies was performed as previously described (Liu et al., 2015a). RNA (5 μg) was treated and the quality and yield were assessed by electrophoresis using the Agilent 2100 bio analyzer (Agilent, Palo Alto, CA USA).

2.3. RNA sequencing (RNA-Seq)

Libraries for RNA-Seq were prepared with Nugen Ovation Human FFPE RNA-Seq Multiplex System. The workflow consists of double-stranded cDNA generation using a mixture of random and poly (T) priming, fragmentation of double stranded cDNA, end repair to generate blunt ends, adaptor ligation, strand selection via nucleotide analog-targeted degradation, In DA-C-mediated adaptor cleavage and PCR amplification to produce the final library. Different adaptors were used for multiplexing samples in one lane. Sequencing was performed on Illumina HiSeq 2500 for a single read 50 run. Data quality check was done on Illumina SAV. Demultiplexing was performed with Illumina CASAVA 1.8.2. The gene expression level was calculated using RSEM software. TPM (transcript per million) was used to normalize the gene expression.

2.4. Quantitative Real-time PCR

Quantitative Real time PCR was performed as previously described (Afifiyan et al., 2017). Briefly, synthesis of first-strand cDNAs was performed with the above mentioned total RNA (1 μg), and random hexamer primers using qScript cDNA XLT cDNA Synthesis SuperMix (Quanta Biosciences, Inc.) following instructions. Real-time PCR was performed using the Fast SYBR Green Master Mix on a StepOnePlus™ Real-time PCR System (Applied Biosystems) with a primer concentration of 300 nM. Primer sequences are as follows: GNA15 (human; NM_002068) forward primer: 5′-GAGAACCGCATGAAGGAGAG-3′, reverse primer: 5′-GAGGATGACGGATGTGCTTT-3′.

Primer sequences and the related gene accession number are listed in Table 1. Reaction conditions consisted of 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s. 60 °C for 30 s. The single PCR product was confirmed with the heat dissociation protocol at the end of the PCR cycles. Human α-tubulin (human; NM_178014) was used as controls to normalize the starting quantity of RNA. Primer sequences are as follows:

Forward: 5′-ACCAGGTGCTGAAAACACAT-3′ reverse: 5′-CTTGAAGCTGAGATGGGAAA-3′. Quantitative values were obtained from the threshold PCR cycle number (CT) at which point the increase in signal associated with an exponential growth for PCR product starts to be detected. The target mRNA abundance in each sample was normalized to its endogenous control level and the relative mRNA expression levels were analyzed using the ΔΔCT method. Reaction of each sample was performed in triplicate.

Table 1
RNA sequencing results on the livers from patients with AH and the controls.

Gene symbol	p-Value(A vs C)	Mean ratio(A vs C)	Fold change(A vs C)	Fold change(A vs C)
PIK3CB	0.0348563	8.59712	8.59712	A up vs C
LIMK2	0.00164893	17.5325	17.5325	A up vs C
GNG2	0.0129814	49	49	A up vs C
GNA15	0.0210469	27.6818	276,818	A up vs C

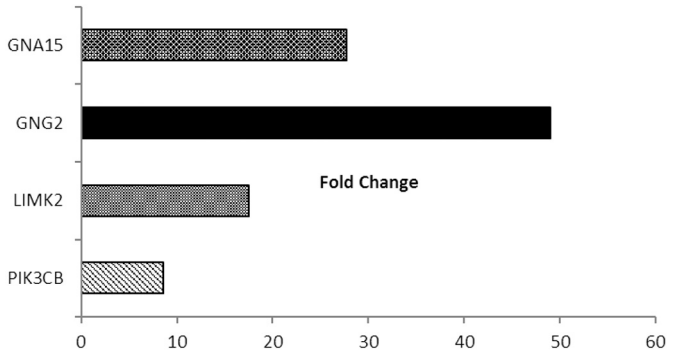

Fig. 1. Four genes, 8 up regulated downstream by IL-8 up regulation were quantitated by RNA seq analysis of liver biopsies from patients with alcoholic hepatitis. (See Table 1 for statistics).

2.5. Immunohistochemical analysis

FFPE tissue slides were double stained for GNA15 or IL-8 and ubiquitin. GNA15 was detected using rabbit polyclonal anti GNA 15 antibody (My BioSource, San Diego CA). The second antibody used was donkey anti rabbit Alexa Fluor 488 (Jackson laboratories West Grove PA). IL-8 was detected using mouse anti IL-8 antibody (LSBio. Seattle, WA). The second antibody used was donkey anti mouse Alexa Fluor 488, (Jackson laboratories West Grove PA). Ubiquitin was detected using mouse anti Ubiquitin antibody (EMD Millipore Corp. Temecula CA.). Donkey anti mouse Alexa Fluor 594 (Jackson Labs, West Grove, PA) was the second antibody used for ubiquitin. All slides were stained with the nuclear stain DAPI (Molecular Probes, Eugene, OR). The fluorescence intensity technology of the stained protein of interest was viewed using a Nikon 800 fluorescent microscope with three filters (FITC-green, Texas Red, and Tri-Color) (Liu et al., 2014). Myeloperoxidase was stained using a rabbit polyclonal antibody (Agilent Technologies, Carpenteria, CA) and viewed by light microscopy.

2.6. Statistical analysis

Statistical significance was determined using the *t*-test and One Way ANOVA test with SigmaStat software. $P < 0.05$ was considered as a statistically significant difference. All data was presented as fold increase.

3. Results

IL-8 and CXCR2 expression were up regulated in the liver biopsies from patients who had alcoholic hepatitis and was reported previously (Liu et al., 2015b). Downstream from IL-8, PIK3CB, GNG2 and LIMK2