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New Markers to Explore Spontaneous Bacterial Peritonitis

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Abstract

Spontaneous Bacterial Peritonitis (SBP) is an ascitic fluid infection without a definitive, surgically treatable, intra-abdominal source, accounting for 10 to 30% of cirrhotic patients with ascites. To explore new methods of diagnosing Spontaneous Bacterial Peritonitis (SBP), we prospectively studied 19 Acute-on-Chronic Hepatitis B Liver Failure (ACHBLF) patients with or without Spontaneous Bacterial Peritonitis and 17 Hepatitis B Virus (HBV)-related Liver Decompensated Cirrhosis patients with or without Spontaneous Bacterial Peritonitis. Patients were separated into two groups: SBP group (n=14), non SBP group (n=22). A paracentesis of ascetic fluid was performed. The levels of 40 cytokines in ascetic fluid were measured by using RayBio® Human Cytokine Antibody Array. The results indicated that the levels of RANTES, EOTAXIN EOTAXIN-2, IL-1βand MIP-1αwere higher in the ascitic fluid of the SBP group. An increase in cytokine production in the ascitic fluid of patients with SBP may provide new markers to diagnose SBP more easily and accurately.

Keywords: Antibody array; Spontaneous bacterial peritonitis; Hepatitis B Virus; Cytokines; Diagnose

Introduction

Spontaneous Bacterial Peritonitis (SBP) is an ascitic fluid infection without a definitive, surgically treatable, intra-abdominal source, accounting for 10 to 30% of cirrhotic patients with ascites [1]. Patients with SBP have an increased risk of developing sepsis, multiple organ failure and dying [2-4], although detection for this disease has increased greatly, ranging from 20% to 40%. In addition, mortality rates 1 to 2 years after an episode of SBP have been reported to be 50-70% and 70-75%, respectively [5]. The prognosis of SBP is closely related to a prompt and accurate diagnosis, and a delay in diagnosis and treatment exposes patients to an increased risk of death [6,7].

A diagnosis of SBP is defined as >250 neutrophils/mm^3 in the ascitic fluid not due to secondary bacterial peritonitis (e.g. hemorrhagic ascites, ascites from pancreatitis or tuberculosis peritonitis, ascites secondary to mycobacterial or fungal peritonitis, and ascites secondary to carcinomatosis) [8]. A number of factors associated with poor diagnosis have been identified in patients with decompensated cirrhosis. Since hypersplenism and suppressed immunity are usually present in patients with cirrhosis [9], the symptoms and signs are frequently absent in SBP patients [1]. The polymorphonuclear neutrophil (PMN) levels in the ascites of those patients are not necessarily elevated during abdominal infection. Besides, PMN count in ascitic fluid is dependent on diuretics and/or other modulations of the ascitic volume. The range of PMN in truly non-infected ascites (i.e. the ascitic PMN count that is clinically relevant for the patients) is not known. Moreover, SBP caused by Gram-positive cocci has been reported frequently to have a PMN count below the threshold of 250/mm., so the diagnosis of SBP has unavoidable problems. Our aim is to find a new way to diagnose SBP.

Despite ascitic fluid bacterial concentrations being low in patients, the ascitic fluid inflammatory responses are markedly stimulated [10]. Several alterations in the inflammatory and immune responses have been described in cirrhotic patients [11]. These alterations could limit the efficacy of inflammatory or immune systems as an adequate response to the infection. Among these mechanisms, cytokines play an important role, being released to ascites in response to liver injury. They have been proposed to be important in the development of ascites. In SBP patients however, the cytokines have not been fully studied to determine their exact role within the patients inflammatory or immune systems. In this study, we measured the ascitic fluid levels of 40 cytokines expressions in the ascitic fluid (AF) of acute-on-chronic hepatitis B liver failure (ACHBLF) patients and hepatitis B virus (HBV)-related liver decompensated cirrhosis patients either with or without spontaneous bacterial peritonitis and investigated the effects of SBP on 40 cytokine expression levels in ascitic fluid to explore new markers to help to make an SBP diagnosis easier and more accurate.

Methods

Patients

From September 2011 to July 2012, Thirty-six patients (31 males and 5 females with a mean ± SD age of 51.6 ±9.5 years were included in this study. 17 patients had HBV-related liver decompensated cirrhosis according to clinical and laboratory evidence as well as ultrasonographic features. 19 patients had acute-on-chronic hepatitis B liver failure (ACHBLF) patients diagnosed by clinical and laboratory evidence [12]. The characteristics of the patients are presented in Table 1. Exclusion criteria were the following: (1) age <18 or >60 years old, (2) clinical evidence of systemic infection except for SBP, (3) antibiotic treatment during the two weeks before admission, (4) treatment which could modify the determination of cytokines (steroidal or non-steroidal anti-inflammatory or immunosuppressive drugs)

Of the SBP group, none had positive ascitic fluid bacterial culture. Three patients had ascite fluids absolute neutrophil counts higher than 250 per mm^3. Eleven patients had symptoms of peritoneal infection. The total leukocyte and neutrophil counts in both blood and ascites were significantly elevated in the SBP group compared with the non SBP group (Table 2).

Characteristic	Acute-on-chronic hepatitis B liver failure n=19	HBV-related liver decompensated cirrhosis n=17	P value
Age (years)	48.63 ± 6.62	55.41 ± 11.24	0.04
Sex			
male	18.00000	14	
female	1.00000	3	
Blood/serum			
DNA(IU/ml)	899818.8 ± 2324591.56	3350642 ± 9341447.43	0.31
ALT(U/L)	98.47 ± 121.59	73.29 ± 99.78	0.51
AST(U/L)	130.11 ± 97.06	109.65 ± 163.99	0.65
TB(umol/L)	453.95 ± 198.67	94.61 ± 134.85	<0.01
PTA(%)	28.68 ± 6.40	63.12 ± 23.58	<0.01
INR	2.72 ± 0.65	1.46 ± 0.34	<0.01
AFP(ng/ml)	64.04 ± 157.36	731.20 ± 2942.51	0.36
WBC （10E9/L）	6.88 ± 4.46	5.59 ± 4.04	0.37
N （%）	66.55 ± 13.24	61.71 ± 13.27	0.28
HGB(g/L)	94.26 ± 17.80	99.59 ± 14.65	0.34
PLT （10E9/L）	79.84 ± 39.91	120.18 ± 75.37	0.06
Ascitic fluid			
WBC （10E6/L）	451 ± 1450.60	4702.59 ± 18870.43	0.37
N(%)	31.94 ± 24.92	32.83 ± 24.76	0.97
RBC （10E6/L）	12031.26 ± 41396.85	6177.94 ± 21639.35	0.61
GL(mmol/L)	7.54 ± 2.19	8.10 ± 3.31	0.55
TP(g/L)	16.42 ± 11.22	12.65 ± 9,73	0.29
LDH （U/L）	83.11 ± 94.41	301.24 ± 997.06	0.35

Aspartate transaminase (AST), alanine aminotransferase (ALT), total bilirubin (TB), prothrombin activity (PTA), international normalized ratio (INR), α-fetoprotein (AFP), platelet count (PLT), hemoglobin (HGB), white blood cells (WBC), percentage of neutrophilic granulocyte (N), red blood cells (RBC), glucose levels (GL), total protein (TP), Lactate dehydrogenase (LDH) and Serum HBV DNA (DNA) were measured by a quantitative polymerase chain reaction assay (DaAn Diagnostics assay and Roche Amplicor, limit of detectability of 100 IU/mL; Roche Diagnostics, Basel, Switzerland) after admission.

Table 1: Characteristics of acute on chronic liver failure patients and hepatitis B virus-related liver decompensated cirrhosis patients

Diagnostic paracentesis was performed on all patients at the time of admmission. Informed consents were obtained from all patients. Paracentesis was carried out the day the patients were admitted using a 20-gauge sterile needle under local anesthesia with lidocaine. After withdrawal from the abdomen, the skin needle was replaced with a sterile needle to minimize contamination by the skin. Ascites fluids were collected under sterile conditions, and the obtained peritoneal fluids were seeded in aerobic and anaerobic blood culture bottles and cultured with an automated Versa TREK culture system at the patient's bedside. Other samples of ascitic fluids were sent to the laboratory and examined for white blood cells, red blood cells, and levels of proteins and glucose. Finally, remaining peritoneal fluids were centrifuged at 1000 rpm for 15 min and supernatants were stored at -80°C until assayed.

All patients were divided into two subgroups as follows: those with an ascitic fluid absolute neutrophil count higher than 250 per mm3 or /and positive ascites fluids culture and/or having any signs or symptoms of peritoneal infection, abdominal pain, rebound tenderness,temperature above 38°C or below 36.5°C, chills, abdominal tenderness suggestive of peritonitis, developing or worsening hepatic encephalopathy, gastrointestinal bleeding within the last 15 days, acute renal failure (defined by an increase in the serum creatinine level to above 133 μmol/L) and arterial hypotension (systolic arterial pressure below 80 mmHg) were allocated to the "SBP group". Those with an ascites absolute neutrophil count lower than 250 per mm^3 and negative ascitic fluid culture were allocated to the"non SBP group".

Parameter	SBP group n=14	non SBP group n=22	P value
Age （years）	50.29 ± 7.03	52.82 ± 10.97	0.05
Sex			
male	13	18.00000	
female	1	4.00000	
Blood/serum			
DNA(IU/ml)	88074.14 ± 176261.26	3310201 ± 8332760.094	0.17
ALT(U/L)	59.29 ± 39.48	103.95 ± 136.93	0.04
AST(U/L)	124.143 ± 104.30	118.09 ± 148.31	0.91
TB(umol/L)	420.77 ± 249.56	197.39 ± 209.69	0.64
PTA(%)	31.64 ± 11.58	53.41 ± 26.25	0.02
INR	2.52 ± 0.92	1.80 ± 0.63	0.02
AFP(ng/ml)	20.78 ± 43.29	606.29 ± 2582.24	0.39
WBC （10E9/L）	9.04 ± 5.40	4.51 ± 1.97	<0.01
N （%）	71.46 ± 11.37	59.68 ± 12.57	<0.01
HGB （g/L）	89.29 ± 10.37	101.55 ± 17.87	0.10
PLT （10E9/L）	110.57 ± 72.70	91.45 ± 54.48	0.64
Ascitic fluid			

WBC （10E6/L）	6153.07 ± 20726.75	107.73 ± 68.46	<0.01
N(%)	44.36 ± 27.76	24.38 ± 18.71	<0.01
RBC （10E6/L）	19719.14 ± 51828.80	2615.95 ± 7630.10	0.68
GL(mmol/L)	7.43 ± 3.6	8.05 ± 1.93	0.24
TP(g/L)	18.19 ± 11.66	12.38 ± 9.40	0.23
LDH（U/L）	392.29 ± 1092.09	54.91 ± 26.55	0.01
Aspartate transaminase (AST), alanine aminotransferase (ALT), total bilirubin (TB), prothrombin activity (PTA), international normalized ratio (INR), α-fetoprotein (AFP), platelet count (PLT), hemoglobin (HGB), white blood cells (WBC), percentage of neutrophilic granulocyte (N), red blood cells (RBC), glucose levels (GL), total protein (TP), Lactate dehydrogenase (LDH) and Serum HBV DNA (DNA) were measured by a quantitative polymerase chain reaction assay (DaAn Diagnostics assay and Roche Amplicor, limit of detectability of 100 IU/mL; Roche Diagnostics, Basel, Switzerland) after admission.			

Table 2: Characteristics of "SBP group" and "non-SBP group"

Laboratory procedures

Determination of cytokine concentration in ascitic fluid was performed using the commercially available RayBio® Human Cytokine Antibody Array kit from RayBiotech, Inc (RayBiotech, Inc., Guangzhou, China). Blocking, incubation and preparation for detection were done according to the manufacturer's instructions. The signals were imaged using an Axon Gene Pix laser scanner using the "green" channel with an excitation frequency of 532 nm.

Statistical analysis

Data are presented as mean ± Stand deviation (SD). All statistical analyses were performed using the SPSS software package (Version 13.0; SPSS, China Guangzhou). Non-categorical variables were compared by the Mann-Whitney U test. Two-sided *p*-values <0.05 were considered statistically significant.

Results

No significant differences were found between patients with acute or chronic liver failure and decompensated cirrhosis in regards to age, sex or levels of DNA, ALT, AST, AFP, WBC, N, HGB, PLT, RBC, FGL, FTP and LDH. (all$p$>0.05), Meanwhile, the mean TB, PTA and INR levels were significantly higher in patients with acute or chronic liver failure than those with decompensated cirrhosis(p<0.05)(Table 2).

The Human Cytokine Antibody Array indicated that 12.5% (5) of the 40 cytokines evaluated were at least1.5-fold up regulated. Expression of RANTES, EOTAXIN-2 and MIP-1a were up regulated in the SBP group compared with the non-SBP group (Table 3).

Cytokine	**SBP group n=14**		**non SBP group n=22**		**Fold change**
	mean	SD	mean	SD	infected/non
EOTAXIN-2	673	768.10	284	184.19	2.29
RANTES	662	1118.95	199	206.49	1.97
M-CSF	48	32.90	66	35.03	0.68
IP-10	3252	1498.22	2,800	1623.06	1.26
IFN-g	112	17.35	120	21.64	0.90
IL-12 p40	34	16.14	42	21.09	0.74
EOTAXIN	932	991.02	646	402.67	1.51
IL-1β	82	205.44	28	35.91	3.04
IL-17	60	13.06	68	25.32	0.89
IL-2	113	19.68	121	20.57	0.92
MCP-1	12,688	6547.10	9,903	7785.99	1.34
GM-CSF	104	12.02	109	15.08	0.95
s TNF RI	10,297	2126.70	9,555	1988.34	1.06
PDGF-BB	20	8.75	25	17.45	0.74
MIP-1d	854	461.65	1,051	664.39	0.77
IL-16	128	173.91	90	41.37	0.93
GCSF	109	16.89	114	18.01	0.94
IL-13	102	12.97	108	22.29	0.95
TNF-a	150	16.71	157	26.03	0.95
IL-12 p70	117	84.62	98	60.42	1.21
IL-4	17	8.18	22	24.12	0.77
IL-3	82	11.82	88	31.27	0.93
IL-7	78	14.40	74	16.79	1.04
MIP-1α	110	239.70	72	71.27	1.59
IL-1α	136	98.24	120	63.11	1.15
s TNF RII	4,681	1477.71	4,365	1646.29	1.08
TIMP-2	4,697	834.02	4,834	872.24	0.98
IL-10	397	303.66	473	593.30	0.81
IL-8	2,802	2971.99	2,309	4220.55	1.27
TNF-b	168	16.44	171	27.36	0.98
I-309	9	6.52	11	12.89	0.90
MCP-2	82	101.60	103	240.79	0.83
MIG	191	79.75	205	192.22	0.88
IL-6sR	7,376	1904.76	7,180	2997.34	1.03
IL-15	101	14.36	100	23.25	1.01
IL-6	11,050	15872.14	12,085	16508.31	0.72
MIP-1β	3,168	3856.66	3,388	3859.64	0.98
TGF-β1	161	19.95	163	34.62	0.99
IL-11	64	12.55	64	11.39	1.00

ICAM-1	2,944	2333.07	2,949	3444.19	1.00

Table 3: Cytokine levels in ascites fluids in SBP group" and "non-SBP group"

Discussion

The present study assessed cytokine expression in ascitic fluid of the SBP group and non-SBP group. Compared to the non-SBP group, the SBP group showed statistically significant increased concentrations of RANTES,EOTAXIN, EOTAXIN-2, IL-1b and MIP-1a (fold change>1.5).

RANTES/CCL5 has significant chemotactic effects on monocytes/ macrophages, T cells and NK cells and it can modulate the function of effector cells [13]. It also exhibits an inflammatory effect by increasing the numbers of local infiltrating monocytes, macrophages, and neutrophils. Previous studies have shown that hepatic RANTES levels increased significantly and the increases were parallel to the increases of the severity of the hepatitis from mild to severe hepatitis [14]. The elevation of hepatic RANTES/CCL5 may be caused by hepatitis B Virus and TNF-α. However, our study does not show higher levels of TNF-α in ascites. Further study is needed to investigate the mechanisms involved.

Eotaxin/chemokine [c-c motif] ligand 11 (CCL11) was originally described as the main chemotactic and activating factor for eosinophils, exerting its chemotactic effects through the chemokine receptor-3 (CCR3) [15]. Eotaxin / Eotaxin-2 are selectively chemo-attractants for the following cells: eosinophils, basophils, Th2 T cells, mast cells, and certain subsets of dendritic cells. Intradermal injection of eotaxin-2 in humans induced infiltration of eosinophils and other inflammatory cells but had little effect on neutrophils [16]. In our study, we did not measure the cell counts of eosinophils or basophils in ascites, and this requires further study.

IL-1βis a multifunctional cytokine that plays a critical role in inflammation, immunity and antiviral response to a variety of diseases. The levels of IL-1β in the serum or within the liver are markedly elevated in the patients with chronic liver diseases [17]. In our study, ascitic levels of IL-1β were highly elevated. A possible mechanism involved in SBP patients could be post exposure of macrophages and dendritic cells to lipopolysaccharide (LPS), which binds to TLR4 and acts as a pathogen-associated molecular pattern, Transcription factor NF-κB can induce IL-1βexpression in ascites [18].

MIP-1αexpression is induced in macrophages by LPS [19], which may be a mechanism involved in the ascites of SBP patients.

SBP is a severe complication of end-stage liver disease, and can cause a high mortality rate unless diagnosed and treated early. Patients who develop SBP may have serum and ascitic fluid characteristics that are different from those who do not develop the infection. SBP is associated with increased cytokines levels. IL-6 has been identified as an important mediator of the synthesis of acute phase reactants and this cytokine also plays a possible role in regulating TNF-α action [20]. Unfortunately, no statistically significant levels of IL-6 or TNF-α in the infected group, either with or without SBP, were observed in our study or between the infected group and non-infected group.

In our study, we found 5 novel cytokines elevated in patients with SBP compared to patients without SBP. Our study also showed elevated levels of anti-inflammatory molecules in those patients with bacterial ascites and elevated pro-inflammatory molecules. That could be interpreted as a protection mechanism in those patients with higher levels of pro-inflammatory compounds. These cytokines may provide additional methods to diagnose patients with SBP, but we did not determine the exact concentration range in ascites to diagnose SBP. As such, further study is necessary to define the exact range by which SBP can be identified and diagnosed in the clinical setting.

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