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Research paper

Comparative proteomic analysis of CD34$^+$ cells in bone marrow between severe aplastic anemia and normal control

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ABSTRACT

Severe aplastic anemia (SAA) is an autoimmune disease with destruction of hematopoietic cells by activated T lymphocytes. However, the precise mechanism of cytotoxicity T cells recognizing and attacking CD34$^+$ cells remains unclear. Here, we investigated the proteome of CD34$^+$ cells in SAA patients to further explore the pathogenesis of SAA. CD34$^+$ cells from 29 SAA patients and 20 health controls were isolated by magnetic activated cell sorting. The protein of CD34$^+$ cells were examined by iTRAQ labeling combination of multidimensional liquid chromatography and tandem mass spectrometry. A total of 156 differential expression proteins in CD34$^+$ cells were identified. Compared with health controls, 53 proteins were up-regulated and 103 proteins were down-regulated in SAA patients. Specifically, abnormal expression of proteasome subunits, histone variants, dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit (DAD1) and ATPase inhibitor, mitochondrial isoform 1 precursor(IF1) may relate to the hyperfunction of immune responses and excessive apoptosis of SAA CD34$^+$ cells.

1. Introduction

Severe aplastic anemia (SAA) is a kind of acquired bone marrow failure syndromes that features severe pancytopenia and high mortality. The incidence of SAA is 7.4/100 million in China. In most cases, SAA is an autoimmune disease with destruction of hematopoietic cells by the activated T lymphocytes [1]. Bone marrow hematopoietic stem and progenitor cells have been confirmed to be the target cells of T cells and Th1 type lymphokine in SAA. CD34$^+$ cell numbers, which were correlated with primitive hematopoietic cell numbers, were markedly diminished in SAA by the long-term culture-intiating cell assay [2]. The myelosuppressive cytokines, such as tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), induced the apoptosis of CD34$^+$ cells by way of Fas/FasL, TRAIL, p38 MAPK in vitro [3–5]. Meanwhile some researchers have found that AA bone marrow CD34$^+$ cells expressed high levels of Fas antigen in vivo [6]. The analysis of transcriptome of CD34$^+$ cells in AA bone marrow showed that a large number of gene implicated in apoptosis and cell death were markedly increased, and negative proliferation control genes also had increased activity, and cytokine/chemokine signal transducer genes, stress response genes, defense/immune response genes were up-regulated, conversely, cell cycle progress-enhancing genes showed low expression [7]. However, the precise mechanism of cytotoxicity T cells (CTL) recognizing and attacking CD34$^+$ cells remains unclear. Proteomics research, which is widely used in the life sciences, has promoted the analysis of the molecular mechanisms of disease at the protein level. Our study used iTRAQ (isobaric tags for relative and absolute quantitation) technology to detect differentially expressed proteins of bone marrow CD34$^+$ cells between SAA patients and normal controls to identify the molecular mechanism of SAA.

2. Materials and methods

2.1. Patients

Twenty-nine patients with SAA in remission were identified, who were hospitalized in the Hematology Department of General

Abbreviations: SAA, severe aplastic anemia; DAD1, dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit; IF1, ATPase inhibitor, mitochondrial isoform 1 precursor; TNF-α, tumour necrosis factor-alpha; IFN-γ, interferon-gamma; CTL, cytotoxicity T cells; iTRAQ, isobaric tags for relative and absolute quantitation; IST, immunosuppressive therapy; PNH, paroxysmal nocturnal hemoglobinuria; BSA, Bovine Serum Albumin; mDCs, myeloid dendritic cells; ATG, Antithymocyte Globulin; CsA, Cyclosporine A; MHC, major histocompatibility complex; PSMB7 proprotein, proteasome subunit beta type-7 proprotein; PSMA2, proteasome subunit alpha type-2; ROS, reactive oxygen species.

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Hospital Tianjin Medical University from January 2014 to February 2015. All remission cases were after immunosuppressive therapy (IST). They all left the infusion of blood products, and their white blood cells counts were normal, of which the proportion of neutrophile granulocytes were normal again. Patients were screened for cytogenetics, and they also were screened for paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry using anti-CD55 and anti-CD59 antibodies. Patients were excluded if they had abnormal chromosomes or PNH clones. The Patients' features were listed in Table 1. There were twenty healthy volunteers in the normal control group which were listed in Table 2. Their routine examination of blood were all normal completely. The study was approved by the Ethics Committee of the Tianjin Medical University. Informed written consent was obtained from all patients or their parents in accordance with the Declaration of Helsinki.

2.2. Isolation and purification of bone marrow $CD34^+$ cells

Twenty milliliters fresh human bone marrow was collected from all cases and controls, in all of whom no infection had been observed and no blood products had been infused for previous three days. Bone marrow mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque Plus solution (Amersham Bioscience, Uppsala, Sweden). The purified $CD34^+$ cells were subsequently obtained by CD34 MicroBead Kit (Miltenyi Biotech, German). After isolation, the purity of $CD34^+$ cells were checked on a routine basis by multiparameter flow cytometry (BD Biosciences) using CD34-FITC-conjugated monoclonal antibodies (from Becton Dickinson, San Diego, CA, USA). Flow cytometry data acquisition and analysis were carried out using the Cell Quest software program (Version 3.1, Becton Dickinson).

2.3. Protein extraction and quantitation

After washing twice in cold phosphate-buffered saline, the collected cells were lysed in lysis buffer (2 M thiourea, 7 M urea and 0.1% CHAPS) and extracted thoroughly with ultrasonication (60 s, 0.2 s on, 2 s off, amplitude 22%). After that, stationary extract for 30 min at room temperature and then centrifuged at 15,000*g* for 20 min at 4 °C and collected the supernatant. The protein concentration was determined by Bradford assay using BSA as standard. The left samples were stored at −80 °C.

Table 2
Characteristics of normal controls.

Case	Age/Sex	WB ($\times 10^9$/L)	Hemoglobin (g/L)	Platelet ($\times 10^9$/L)	RET%
1	20/M	5.91	126	223	0.78
2	19/M	7.26	130	152	0.92
3	27/F	4.21	127	258	1.41
4	18/F	8.73	129	117	1.29
5	27/M	6.12	137	246	1.46
6	22/M	5.24	141	183	1.17
7	26/M	4.75	122	273	1.08
8	32/M	7.19	139	192	1.68
9	16/F	5.27	128	146	1.58
10	11/F	5.15	140	245	1.35
11	14/F	8.49	142	189	0.86
12	39/M	4.36	137	226	1.27
13	31/M	7.92	126	159	1.19
14	18/M	6.81	141	238	1.06
15	19/F	5.86	125	139	0.97
16	45/M	5.58	142	255	1.69
17	41/M	7.67	136	123	0.89
18	50/M	6.13	145	232	0.95
19	39/M	5.61	121	251	1.39
20	22/F	7.25	139	206	1.22

2.4. Trypsin digestion and iTRAQ labeling

Proteins were dissolved, denatured, alkylated and digested with trypsin (Sigma) at 37 °C for 18 h or overnight. To label peptides with iTRAQ reagent (Foster City, CA), 1 unit of label (defined as the amount of reagent required to label 100 μg protein) was

Table 1
Characteristics of SAA patients.

Case	Age/Sex	Granulocyte ($\times 10^9$/L)	Hemoglobin (g/L)	Platelet ($\times 10^9$/L)	RET%	Abnormal chromosome	Therapy
1	49/M	0.12	91	12	0.12	Absence	ATG+CsA
2	50/M	0.16	90	2	0.25	Absence	ATG+CsA
3	28/F	0.47	62	17	0.11	Absence	ATG+CsA
4	20/M	0.39	58	11	0.4	Absence	ATG+CsA
5	18/M	0.38	46	13	0.3	Absence	ATG+CsA
6	37/M	0.35	65	4	0.32	Absence	ATG+CsA
7	35/M	0.24	91	14	0.62	Absence	ATG+CsA
8	28/M	0.14	66	9	0.22	Absence	ATG+CsA
9	17/M	0.05	94	15	0.19	Absence	ATG+CsA
10	19/M	0.38	81	8	0.23	Absence	ATG+CsA
11	8/F	0.17	80	18	0.12	Absence	ATG+CsA
12	10/F	0.46	79	12	0.43	Absence	ATG+CsA
13	24/M	0.07	68	10	0.31	Absence	ATG+CsA
14	15/M	0.04	75	17	0.15	Absence	ATG+CsA
15	41/F	0.28	62	9	0.09	Absence	ATG+CsA
16	26/M	0.31	91	3	0.26	Absence	ATG+CsA
17	24/M	0.43	69	12	1.45	Absence	ATG+CsA
18	18/M	0.02	81	5	0.09	Absence	ATG+CsA
19	15/F	0.39	53	6	0.5	Absence	ATG+CsA
20	12/F	0.37	78	19	0.13	Absence	ATG+CsA
21	17/F	0.26	93	3	0.07	Absence	ATG+CsA
22	23/F	0.26	57	9	0.08	Absence	ATG+CsA
23	17/F	0.11	71	11	0.18	Absence	ATG+CsA
24	19/M	0.13	89	7	0.51	Absence	ATG+CsA
25	20/M	0.25	63	8	0.46	Absence	ATG+CsA
26	47/F	0.33	75	6	0.29	Absence	ATG+CsA
27	32/F	0.19	60	3	0.15	Absence	ATG+CsA
28	24/M	0.05	45	5	0.06	Absence	ATG+CsA
29	16/F	0.45	78	10	0.21	Absence	ATG+CsA

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