Original Article

A Study on the Mechanism of Anemia and Leukopenia in Liver Cirrhosis

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To elucidate the mechanism of anemia and leukopenia in the patients with liver cirrhosis, we investigated hematopoietic progenitor cell contents in their bone marrow, and the effects of their sera and blood mononuclear cells on hematopoietic progenitors from normal subjects. While there was no significant difference in the number of marrow CFU-E and BFU-E between the patients with liver cirrhosis and normal subjects, the number of marrow CFU-C was significantly reduced in liver cirrhosis patients. Patients' sera suppressed in vitro colony formation of normal CFU-E, BFU-E, and CFU-C, and the degree of suppression was well correlated with severity of anemia or granulocytopenia. In vitro colony formation of normal hematopoietic progenitor cells was not affected by blood mononuclear cells from liver cirrhosis patients. These results indicate that the appearance of humoral inhibitor(s) of hematopoietic progenitors plays a role in the development of anemia and granulocytopenia in liver cirrhosis.

Key Words: Liver cirrhosis, Anemia, Leukopenia, CFU-E, BFU-E, CFU-C.

Anemia and leukopenia are frequent manifestations of chronic liver diseases, particularly, liver cirrhosis[1, 2]. Approximately 40 to 70% of the patients with liver cirrhosis are found to have anemia[1,3]. It can be caused by acute and/or chronic bleeding from the upper gastrointestinal tract and hemorrhoid, but it is often observed despite of no obvious bleeding. As the developing mechanism of anemia in such patients, folate deficiency[4], hypersplenism[5] and hemolysis such as spur cell anemia[6] have been described. Granulocytopenia in liver cirrhosis has been ascribed to increase of marginal granulocytic pool and appearance of splenic inhibitory factor of granulopoiesis[7].

We investigated hematopoietic progenitor cell contents in the bone marrow of the patients with liver cirrhosis, and the effects of their sera and blood mononuclear cells on hematopoietic progenitors from normal subjects to elucidate the mechanism of anemia and granulocytopenia in this disorder.

MATERIALS AND METHODS

1. Patients

Twenty-five patients with liver cirrhosis diagnosed by laparoscopy and liver biopsy were studied. They consisted of 6 females and 19 males, their age ranging between 36 and 72 years, and the duration of the disease was one month to 60 months after diagnosis. One of the patients had hepatitis B virus-associated liver cirrhosis; 11 had alcoholic liver cirrhosis and 13 had liver cirrhosis of unknown origin. Patients who had histories of apparent hemorrhage, positive stool occult blood, iron deficiency, receiving blood transfusion and pyrexia were excluded from this study. The patients were divided into two groups according to the hemoglobin concentration; anemic group (Hb concentration $<$ 12g/dl for males, $<$ 11g/dl for

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females, 12 patients) and non-anemic group (13 patients). The patients were also divided into two groups according to the granulocyte count; granulocytopenic group (granulocytes $< 2000/mm^3$, 11 patients) and non-granulocytopenic group (14 patients). Ten normal subjects were studied as controls.

2. Cell and Serum Preparation

About 2 to 4 ml of heparinized bone marrow aspirate was diluted with the same volume of alpha-medium (Flow, U.S.A.). The mononuclear cells were separated over a Ficoll-Hypaque gradient (Lymphoprep SG 1.077, Nycomed AS, Norway), washed three times with enough volume of alpha-medium, and were prepared for culture at a final concentration of 2×10^5 cells/ml.

Mononuclear cells were separated from 20 ml of peripheral blood collected in heparinized syringe, using the same method as for preparation of bone marrow mononuclear cells. Cell counting was made by a Coulter Counter Model ZM (Coulter Electronics U.S.A.).

All sera were heat-inactivated at 56℃ for 30 min, sterilized by passing through membrane filters (Millipore filter, Millex, U.S.A.), and stored at −20℃ until use.

3. Culture of Hematopoietic Progenitors

In vitro CFU-E (erythroid colony-forming units) and BFU-E (burst-forming units-erythroid) assays were performed using the plasma clot method of Terasawa et al.[8] with minor modifications. In brief, 8×10^4 bone marrow mononuclear cells were inoculated in a culture plate (Falcon, U.S.A.) with 1.0 u/ml (for CFU-E) and 2.0 u/ml (for BFU-E) of erythropoietin (Connaught, Step III, U.S.A.). After seven days (for CFU-E) and 14 days (for BFU-E) incubation at 37℃ in fully humidified atmosphere with 5% carbon dioxide in air, the plasma clot was fixed with 5% glutaraldehyde and stained with benzidine. Cell aggregates consisting of 8 or more benzidine-positive cells were scored as CFU-E derived colonies and those consisting of 50 or more benzidine-positive cells, and three or more cluster aggregates, each containing 8 to 49 benzidine-positive cells, were scored as BFU-E derived colonies.

For CFU-C (granulocyte-macrophage colony forming unit) assay, the monolayer soft agar method by Bradley and Metcalf[9] was used with minor modifications. In brief, 2×10^5 bone marrow mononuclear cells were suspended in 1 ml of culture medium containing 0.3% bacto-agar in a 35mm Falcon petri dish. The leukocyte-conditioned medium prepared from normal human peripheral leukocytes[10], as a source of colony stimulating factor, was added to the culture system at a final concentration of 20%. Duplicate cultures were incubated for 12 days at 37℃ in fully humidified atmosphere with 5% carbon dioxide in air. Aggregates consisting of 50 or more cells were scored as CFU-C colonies.

4. Statistical Analysis

The Student's t-test was used to determine statistically significant difference.

RESULTS

1. Hematopoietic Progenitor Contents in the Bone Marrow

The numbers of CFU-E derived colonies were 180.4 ± 119.2 in the whole patients (mean ± SD, n=15), 173.7 ± 96.5 (n=7) in anemic group, 180.6 ± 153.0 (n=8) in non-anemic group, and 157.2 ± 73.4 (n=10) in control group. There was no significant difference in these four groups. Similarly, BFU-E derived colonies in the four groups were 38.8 ± 26.8 (n=10), 38.6 ± 14.3 (n=5), 39.0 ± 37.5 (n=5), 49.1 ± 35.2 (n=7), respectively and there also was no significant difference in these four groups. By contrast, the number of CFU-C derived colonies were 77.3 ± 46.9 (n=14) in the whole patients and 125.5 ± 45.7 (n=10) in control group. These values were significantly different one from the other ($p < 0.04$). Further, CFU-C colony counts were 39.0 ± 18.7 (n=5) in granulocytopenic group and 98.6 ± 44.4 (n=9) in non-granulocytopenic group ($P < 0.02$) (Fig. 1).

2. Effect of Patients' Sera on Colony Formation of Normal Human Hematopoietic Progenitors

Patients' sera were added to marrow

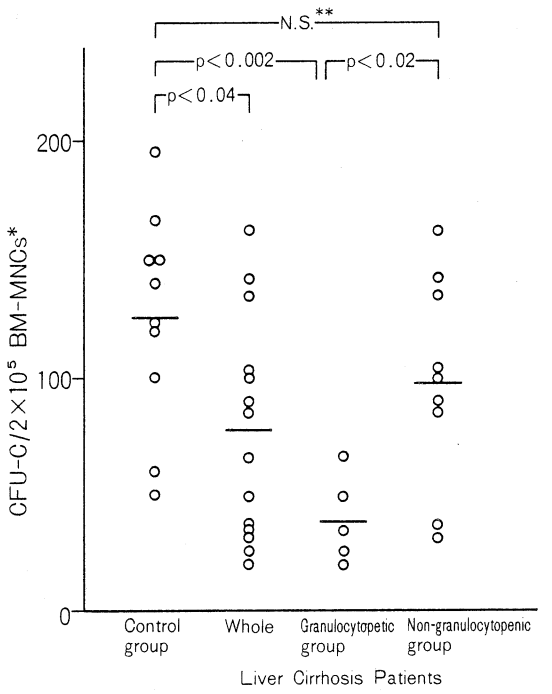

Fig. 1. Colony formation of the bone marrow CFU-C from liver cirrhosis patients and normal subjects.
* Bone marrow mononuclear cells
** Not significant

hematopoietic progenitor culture system of ABO-compatible or type O normal subjects at a final concentration of 10%. This experiment was repeatedly carried out using normal target marrow cells obtained from at least two different donors. The result was expressed as a percentage of the colony count in culture with normal AB serum. Addition of sera from the patients in anemic and granulocytopenic group significantly suppressed in vitro colony formation of normal CFU-E, BFU-E, and CFU-C, respectively. In contrast, no in vitro suppressive effect was observed on normal hematopoietic progenitor cells by addition of sera from the patients in non-anemic or non-granulocytopenic group (Table 1.). Furthermore, the degree of suppression was well correlated with the severity of anemia or granulocytopenia (Fig. 2-4).

3. *Effect of Patients' Peripheral Mononuclear Cells on Colony Formation of Normal Human Hematopoietic Progenitors*

Table 1. Effect of patients' sera on colony formation of normal human hematopoietic progenitors.

	Whole patients	Anemic group	Non-anemic group
CFU-E	85.5±25.9%* (n=25)	68.7±23.0% (n=12)	101.0±17.9% (n=13)
BFU-E	80.0±23.7% (n=22)	65.2±23.7% (n=11)	94.8±11.8% (n=11)
	Whole patients	**Granulocytopenic group**	**Non-granulocytopenic group**
CFU-C	83.2±20.5% (n=25)	71.8±23.3% (n=11)	92.1±12.8% (n=14)

† $p < 0.0012$, †† $p < 0.002$, ††† $p < 0.02$
* The results were expressed as a percentage of the colony count in culture with normal AB serum.

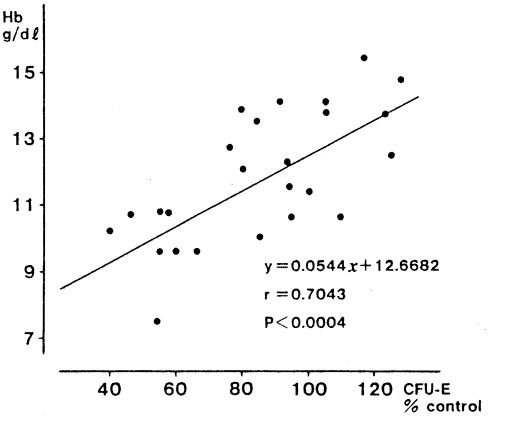

Fig. 2. Effect of patients' sera with liver cirrhosis on colony formation of normal human CFU-E.
Each dot means a percentage of control culture with normal AB serum in each patients.

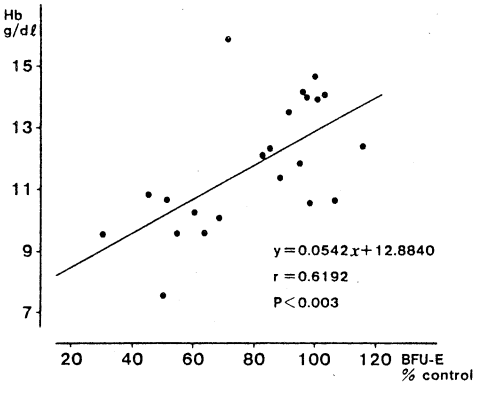

Fig. 3. Effect of patients' sera with liver cirrhosis on colony formation of normal human BFU-E.
Each dot means a percentage of control culture with normal AB serum in each patients.

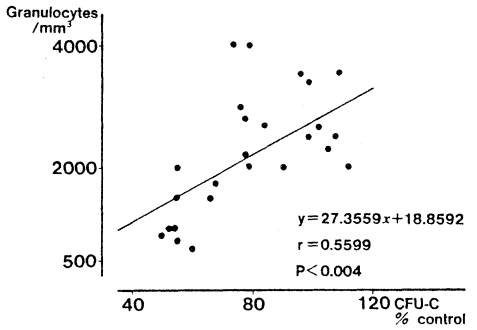

Fig. 4. Effect of patients' sera with liver cirrhosis on colony formation of normal human CFU-C.
Each dot means a percentage of control culture with normal AB serum in each patients.

Table 2. Effect of patients' peripheral mononuclear cells on coloy formation of normal human hematopoietic progenitors.

	Whole patients	Anemic group	Non-anemic group
CFU-E	91.0±14.6%* (n=15)	92.5±10.1% (n=8)	89.3±19.3% (n=7) †
BFU-E	95.9±17.5% (n=12)	90.2±20.3% (n=6)	98.3±13.5% (n=6) †
	Whole patients	Granulocytopenic group	Non-granulocytopenic group
CFU-C	113.2±16.2% (n=12)	110.8±22.0% (n=5)	114.9±12.2% (n=7) †

† not significant
* The results were expressed as a percentage of the colony count without addition of mononuclear cells.

Patients' peripheral mononuclear cells were added to marrow hematopoietic progenitor culture system of ABO-compatible normal subjects at ratio 1:1. Addition of patients' peripheral blood mononuclear cells did not influence the colony formations of normal CFU-E, BFU-E, or CFU-C (Table 2.).

DISCUSSION

Anemia is frequently found in the patients with liver cirrhosis despite no obvious bleeding, but its mechanism is not entirely elucidated. Recently, it has been reported that the sera from the patients with rheumatoid arthritis[11], disseminated bronchial cancer[12], chronic renal failure[13], malignant lymphoma[14] and systemic lupus erythematosus[15] suppressed normal hematopoietic progenitors in vitro, and that lymphocytes from aplastic anemia[16], pure red cell aplasia[17] and systemic lupus erythematosus[18] were associated with a part of their hematological abnormalities. It may be inferred that humoral and/or cellular inhibitor(s) are related to a part of pathogenesis of hematological abnormalities in other diseases. In this study, we demonstrated that the sera of liver cirrhosis patients with anemia suppressed in vitro colony formation of normal marrow CFU-E and BFU-E, and the degree of suppression was well correlated with the severity of anemia. Our results indicate that the appearance of humoral inhibitor of erythroid progenitor cells may be one of mechanisms of anemia in liver cirrhosis patients. In our results, no decrease of marrow erythroid progenitors was observed even in the patients whose sera clearly suppressed erythroid colony formation from normal bone marrow. This finding may indicate that the humoral inhibitor of liver cirrhosis patients acts on the later stage of erythroid differentiation, but not directly affects the proliferation of CFU-E and BFU-E themselves.

We also found that the sera of liver cirrhosis patients with granulocytopenia suppressed in vitro colony formation of normal marrow CFU-C, and that the degree of suppression was well correlated with the severity of granulocytopenia. The appearance of this inhibitor may be one of the causes of granulocytopenia in liver cirrhosis. It is not obvious whether or not the serum inhibitory factor found in our study is identical with so-called splenic inhibitor. This CFU-C inhibitory factor may be different from our erythroid inhibitor, since marrow CFU-C significantly decreased in the liver cirrhosis patients where CFU-E and BFU-E remained normal in number. It is speculated that the CFU-C inhibitory factor suppresses proliferation of granulocyto-macrophage progenitor cells in the stage of CFU-C in vitro.

These inhibitory factors demonstrated here have not been reported previously, and will play a role in the development of anemia and/or granulocytopenia in liver cirrhosis. Their chemical property is yet to be elucidated, presently under investigation.

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