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Effect of Hemopoietic Growth Factors on Long-term Marrow Culture From Patients with Aplastic Anemia

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ABSTRACT

Objective: The long-term marrow culture (LTMC) system provides an in-vitro physiological model for the study of stromal cell mediated hemopoiesis in patients with aplastic anemia. The two aspects of hemopoiesis - stromal and stem cell function - can be studied using LTMC. Addition of exogenous hemopoietic growth factors to LTMC can predict, to some extent, the result of administering such factors in-vivo as therapeutic drugs. This work aimed at studying the effect of some different important hemopoietic growth factors on LTMC of aplastic anemia patients.

Design and methods: The plan of this work was to evaluate the effect of SCF, IL3, GM-CSF, and G-CSF on LTMC of 19 newly diagnosed pediatric patients suffering from aplastic anemia. The loss of LTC-IC was assessed weekly in the presence of these different cytokines.

Results: LTMC study has demonstrated severely reduced generation of CFUs in patients with aplastic anemia at diagnosis, with no detectable hemopoiesis beyond 4 weeks of culture in 10/19 cases. There was a highly significant difference between the number as well as the maintenance of CFUs in LTMC between the controls and the aplastic anemia cases ($p<0.0001$).

Conclusion: These results show that hemopoietic growth factors can partially maintain and support the proliferation and differentiation of primitive cells of some cases of aplastic anemia patients for only short period of time in LTMC. Since routinely used concentrations and combinations of hemopoeitic growth factors, early acting as well as differentiating factors are currently not considered one of the regular therapeutic options in aplastic anemia, further studies using escalated concentrations of different combinations of growth factors might be tried to evaluate their exact role. We suggest that the best curative treatment up till now would be allogenic bone marrow transplant (BMT) from normal donor.

Key Words: *Aplastic anemia - Long term marrow culture - Growth factors.*

INTRODUCTION

Patients with aplastic anemia universally demonstrate defective stem cell function in terms of reduced or absent marrow repopulating ability, reflecting a deficiency of long-term culture initiating cells (LTC-ICs). Defects in stromal cell function, as assessed by the ability of aplastic anemia stroma to support normal generation of hemopoietic progenitors, are not common, but may conceal an isolated deficiency of a particular growth factor in some patients due to the overlapping nature of hemopoietic growth factor activities. The stem cell abnormality in aplastic anemia reflects a deficiency in cell numbers, as well as dysfunction in certain cases **[15,19]**.

An increased level of apoptosis in aplastic anemia marrow CD34+ cells exists, and this correlates well with disease severity. LTMC studies demonstrated that more of the hemopoietic cells are nonviable (apoptotic) compared with normal controls, and this correlates with reduced colony (CFU-GM) generation. An increase in apoptosis among primitive hemopoietic cells may contribute to the stem cell defect in aplastic anemia **[2]**.

The principle of LTMC is based on a model for the control of hemopoiesis, namely stromal cell-mediated hemopoiesis. The stromal cells synthesize, secrete and present growth as well as inhibitory factors, provided at the appropriate combinations and concentrations, to the hemopoietic stem cells. The cells within the stromal layer include fibroblasts, fat cells, macrophages and endothelial cells. Subsequently, primitive hemopoietic cells migrate beneath and between the stromal cells. The most immature cells remain within this adherent stromal layer and as they divide and mature, some of the progeny are released into the growth medi-

um; where they can be assessed each week. The most primitive hemopoietic cells within the stromal layer can also be assessed after trypsinisation of the stromal layer. The human LTMC assesses the generation of CFU-GM from marrow cells with repopulation ability (LTC-ICs) for weeks [13].

MATERIAL AND METHODS

Bone marrow sampling:

An important practical consideration for establishing LTMC from patients with aplastic anemia concerns the volume of bone marrow aspirated. Marrow sample heavily contaminated with peripheral blood would produce poorly formed stroma in LTMC; hemopoietic progenitor cell numbers would also be diluted down. In aplastic anemia, where the bone marrow is hypocellular, it is therefore important to aspirate only very small aliquots of bone marrow from at least two sites, in-order to avoid a falsely high rate of non-confluency of stroma formation. This may partly explain the variability in reported rates of non-confluent stroma formation in aplastic anemia, with a higher proportion among those with more severe disease [2].

This study included 19 pediatric patients suffering from aplastic anemia (newly diagnosed) with age ranging between 1.5 and 17 years. They were 17 cases with acquired aplastic anemia (AAA) and two cases with Fanconi anemia. It also included 8 control cases with non-aplastic as well as non-neoplastic disorders.

LTMC assay:

Bone marrow sample of each case was aspirated (0.2 ml) from at least two sites under general anesthesia in the operating theatre under the parents' consent. Aspirated marrow sample was collected in a sterile tube containing 20U preservative-free heparin (Sigma, Saint Fallavier, France). Bone marrow MNCs were isolated on lymphocyte separation medium (Gibco, Life Technologies, U.K) and counted. MNCs (5 $x10^6$) were adjusted and seeded into 25 cm^2 tissue culture flasks (Falcon, Subra, Toulouse, France) in a total volume of 10 ml of complete medium consisting of 1x Alpha medium (Gibco), with 12.5% fetal calf serum (FCS, Gibco), 12.5% horse serum (HS, Gibco), 2mmol/l L-glutamine (Gibco), 10^{-4}mol/l 2-mercaptoethanol (2-ME, Sigma), 0.2mmol/l inositol (Gibco), 20µmol/l folic acid (Gibco) and10^{-6}mol/l freshly dissolved hydrocortisone (Sigma). Early acting hemopoietic growth factors were added to the LTMC: 10ng/ml Interleukin-3 (IL-3, Promega, Southhampton, U.K) and 50ng/ml stem cell factor (SCF, R&D, Abington, U.K); as well as differentiating growth factors: 10ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF, R&D) and 10ng/ml granulocyte colony stimulating factor (G-CSF, R&D). Culture flasks were incubated at 33ºC in a humid atmosphere with 5% CO_2. They were fed weekly by replacement of half of the growth medium containing half of the non-adherent cells with fresh complete medium [13]. LTMCs were maintained for five weeks. The weekly collected media were stained for assessment and counting colony forming units if present.

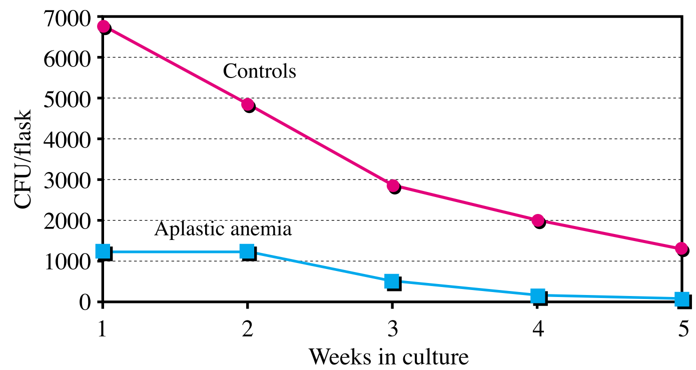
RESULTS

Table (1) shows the mean routine parameters of all cases at diagnosis.

LTMC study has demonstrated severely reduced generation of CFUs in pediatric patients with aplastic anemia at diagnosis, with no detectable hemopoiesis beyond 4 weeks of culture in 10/19 cases (one case with Fanconi anemia and 9 cases with AAA). There was a highly significant difference between the number as well as the maintenance of CFUs in LTMC between the controls and the aplastic anemia cases ($p<0.0001$) (Table 2, Fig. 1). After one week of LTMC, it was possible to observe into the culture the differentiation of round cells. These cells were multiplying in vitro. Apparently they did not follow any differentiation lineage. These cells seemed to persist in an immature state. It was also possible to observe in a cytospin preparation of two cases some megakarocytes as well as eosinophils, these two cases had platelet count >40,000/ cmm (Fig. 2-5)

Table (3) shows comparison of the different laboratory parameters at diagnosis between cases with sustained LTMC and unsustained LTMC beyond 4 weeks. Hemoglobin (HB%) and absolute granulocytic count were the only significantly different parameters between the two groups with p value =0.005, =0.001, respectively (Table 3).

Fig. (1): Generation of colony forming units from the non-adherent layer of long-term bone marrow cultures from patients with aplastic anemia (n=19) compared with controls (n=8). Difference between cases and controls is highly significant (p<0.0001)for whole time period of the culture, i.e. wk 1-5.



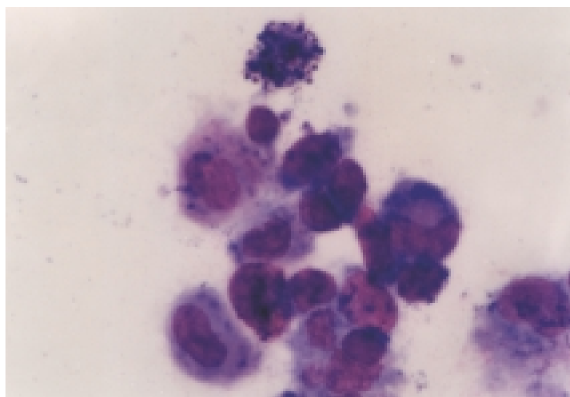


Fig. (2): Colony forming unit containing eosinophil.

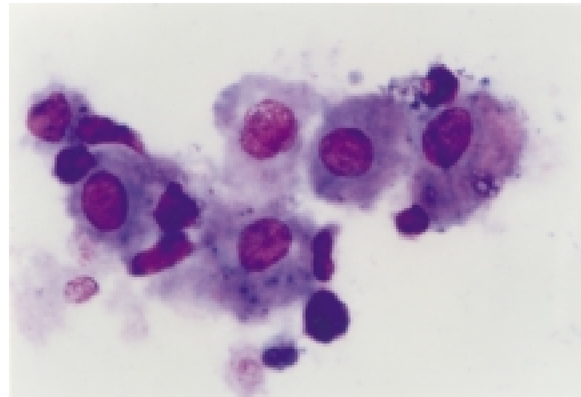


Fig. (3): Stromal cells after 5 weeks of LTMC (obtained by trypsinization).

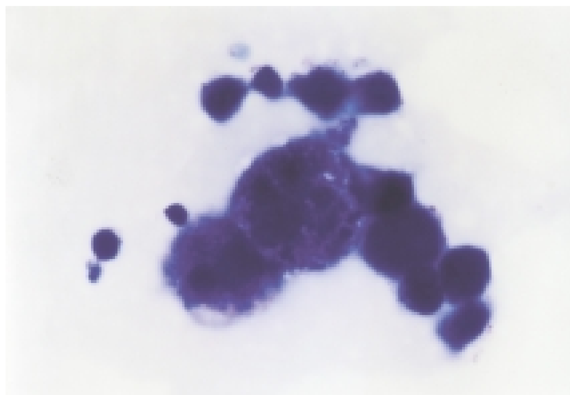


Fig. (4): Two megakaryocytes observed in a cytospin preparation.

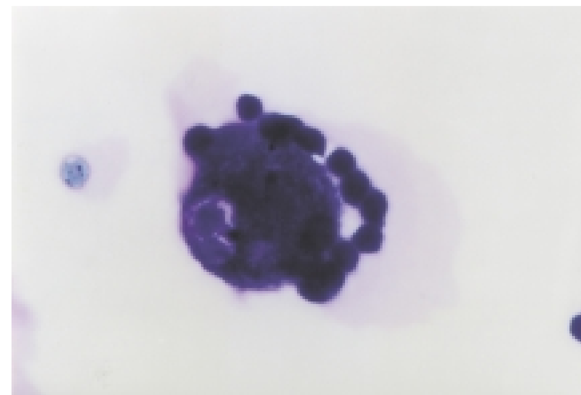


Fig. (5): Megakaryocyte surrounded by MNCs in a cytospin preparation from LTMC.

Table (1): Routine laboratory parameters of all studied cases.

	Age (years)	Hb (gm/dl)	Reticulocytic count (%)	AGC (/cmm)	Platelet count ($x10^3$/cmm)	BM cellularity (%)
Mean	7.3	7.2	0.54	1356.08	27.21	22.86
Std. Dev.	4.6	2.03	0.49	636.94	24.89	13.83
Minimum	1.5	3.6	0.05	150.00	1.00	10.00
Maximum	17	10.8	1.8	4524.00	92.00	40.00

AGC: absolute granulocytic count

Table (2): Correlation study between no. of colony count/plate of LTMC of aplastic anemia cases (n=19) and controls (8) during 5 weeks.

Weeks	Valid no aplastic	Mean aplastic colony/plate	minimum aplastic colony/plate	maximum aplastic colony/plate	St. dev. aplastic	Valid no control	Mean control colony/plate	minimum control colony/plate	maximum control colony/plate	St. dev. control	*p*-value	t-value
1st	19	1257.895	100.000	2500.000	833.544	8	6775.000	5300.000	8500.000	993.910	<0.0001	-14.8519
2nd	19	1235.789	0.000	4000.000	1104.085	8	4856.250	3350.000	5900.000	838.978	<0.0001	-8.2860
3rd	19	515.789	0.000	2600.000	682.531	8	2862.500	1600.000	4600.000	1112.189	<0.0001	-6.7435
4th	19	176.316	0.000	700.000	221.340	8	2002.500	1250.000	3100.000	647.870	<0.0001	-11.0847
5th	19	83.158	0.000	300.000	114.749	8	1312.500	930.000	2200.000	409.207	<0.0001	-12.2858

Table (3): Comparison of different parameters between cases with unsustained (group A) and sustained (group B) LTMC beyond 4 weeks.

Group	Age (years)	Hb (gm/dl)	Reticulocytic count (%)	AGC (/cmm)	Platelet count ($x10^3$/cmm)	BM cellularity (%)
A:						
Mean	7.75	5.65	0.47	554.50	21.67	18.33
Std. Dev.	3.19	1.06	0.34	210.11	15.97	11.69
B:						
Mean	7.0	8.36	0.59	1756.88	32.38	26.25
Std. Dev.	5.72	1.81	0.61	908.17	30.37	15.06
p-value	0.769	0.005	0.663	0.001	0.456	0.290

DISCUSSION

Stem cell proliferation induced by potent cytokines usually leads to a loss of primitive potential through differentiation. In this study, the ability of cytokines to regulate the proliferation and long-term culture-initiating cell (LTC-IC) activity of primitive hemopoietic human bone marrow cells of aplastic anemia patients was evaluated. The loss of LTC-IC was assessed weekly in the presence of different cytokines. LTMC study has demonstrated severely reduced generation of CFUs in pediatric patients with aplastic anemia at diagnosis, with no detectable hemopoiesis beyond 4 weeks of culture in 10/19 cases. There was a highly significant difference between the number as well as the maintenance of CFUs in LTMC between the controls and the aplastic anemia cases ($p<0.0001$).

In a trial to find any relevant factor that could be predictable to sustainability of LTMC, we compared the laboratory parameters of cases with sustained LTMC and unsustained LTMC beyond 4 weeks. We found that the only significant parameters were the HB% and absolute granulocytic count ($p=0.005$, $p=0.001$ respectively).

After one week of LTMC in our study, it was possible to observe into the culture the differentiation of round cells. These cells were multiplying in vitro. Apparently they did not follow any differentiation lineage. These cells seemed to persist in an immature state. The central question left behind is about their origin. The role of these cells must be very critical and the way they react to each other must be relevant. The attempt to give an explanation would be consistent with precursor cells that survive into culture, probably as small lymphocyte morphology, that undergo apoptosis as soon as the micro-environment reaches a trigger point. This phenomenon appears to be quite similar to what has been already described by other studies [3, 4]. It should be very interesting to find out what allows the cells to persist in the immature state.

The addition of exogenous hemopoietic growth factors to LTMC can predict, to some extent, the result of administering such factors in-vivo. It was reported that growth factors such as G-CSF, GM-CSF and IL-3 added to normal LTMC served only to partially maintain the CFU-GM each week of culture but did not prolong the longevity of the cultures, even after continued administration of growth factors [7, 8, 10]. This would suggest that the LTMC system

possess an inherent feedback system, probably involving various inhibitory factors, which function to maintain the normal hemopoietic homeostasis. Other investigators had examined the effect of adding a combination of GM-CSF and IL-3 to LTMC of aplastic anemia patients and demonstrated no further effect to result from the addition of SCF to GM-CSF and IL-3 [11,18].

Other authors investigated the effect of different combinations of hempoietic growth factors in aplastic anemia patients. They demonstrated that although SCF and IL-3 do not require additional cytokines to generate early progenitors from primitive human marrow cells, additional cytokines are required to force terminal differentiation of precursors. Conversely, SCF and IL-3 do not directly affect differentiation, but instead sustain the long-term expansion of early progenitors, which can be induced to produce mature cells by other cytokines such as GM-CSFand G-CSF [12].

In the steady state of normal marrow, for hemopoietic progenitor cells, there is a fine balance between proliferation and differentiation, and apoptosis. Hemopoietic growth factors stimulate their growth and development, but also suppress apoptosis thereby increasing their survival. A deficiency of growth factors would result in increased cell death of stem cells by apoptosis. It has been demonstrated that negative regulatory factors, such as tumour necrosis factor α (TNFα) and interferon γ (IFN γ), upregulate the expression of the Fas antigen (Fas-Ag) on normal marrow CD 34 + cells leading to increased apoptosis [14,20,21]. Increased or aberrant expression of TNFα and IFN γ had earlier been demonstrated in some patients with aplastic anemia. Furthermore, aplastic anemia CD34+ cells were found to show increased expression of Fas-Ag compared with normal cells, and this correlated with increased inhibition of colony growth in-vitro [1,5,16,17].

The possible contribution of apoptosis to the stem cell deficiency/defect seen in LTMC in aplastic anemia has been investigated using flowcytometry (FCM) [8]. The authors analyzed non-adherent LTMC cells each week of culture from 10 aplastic anemia patients and 10 normal controls. The percentage of apoptotic cells in normal LTMC remained constant at 10-20% throughout the life of the culture, with no late increase in apoptosis occurring that might have provided a theoretical mechanism for the termination of normal LTMC. In-contrast, there was an increase in the percentage of apoptotic cells in aplastic anemia LTMC after 2 weeks, which increased further with the life of culture [8].

From this study, we can conclude that hemopoietic growth factors using the routinely used concentrations and combinations can partially support and maintain the proliferation and differentiation of primitive cells of aplastic anemia patients for only short period of time in LTMC. In-addition, further studies to define the exact etiology of cell death in aplastic anemia should be performed. Since routinely used concentrations and combinations of hemopoeitic growth factors, early acting as well as differentiating factors are currently not considered one of the regular therapeutic options in aplastic anemia, further studies using escalated concentrations of different combinations of growth factors might be tried to evaluate their exact role. We suggest that the best curative treatment up till now would be allogenic BMT (however, it can only be offered to a small proportion of patients because of lack of HLA-matched normal sibling donor and the role of matched unrelated donor transplants remains uncertain [6,9]).

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