Haematology and Blood Transfusion Vol. 28
Modern Trends in Human Leukemia V
Edited by Neth, Gallo, Greaves, Moore, Winkler

Studies on the In Vitro Microenvironment in Man *

J. W. Singer and A. Keating

A. Introduction

The recent adaptation of the murine long-term marrow culture system to humans by Gartner and Kaplan [1] has, for the first time, allowed an examination of the characteristics of those cells that form the in vitro, and possibly the in vivo, microenvironment. Both the human and the murine long-term marrow cultures develop characteristic adherent stromal cell layers that consist of cells described by Dexter as "endothelial-like, fat-containing cells and macrophages" [2]. Until recently, only descriptive information was available about marrow stromal cells (MSC) that are required for the support of in vitro hematopoiesis. We have initiated a series of studies to examine certain properties of MSC and their precursors. These studies suggest that MSC are derived from a transplantable stem cell that is multipotent for other hematopoietic elements. However, MSC also share characteristics with mesenchymal cells previously thought to be unrelated to hematopoietic lineages.

B. Studies in Marrow Transplant Recipients

We examined the origin and some characteristics of MSC in long-term marrow cultures derived from samples obtained from patients who had undergone marrow transplantation following ablative therapy for either leukemia or aplastic anemia. Each of the 14 patients studied received marrow from a sibling of the opposite sex. Marrow samples were obtained between days 14 and 490 after transplantation and used to establish long-term cultures. At 4 weeks after culture initiation, when all cultures had confluent stromal cell layers and were producing CFU-C, supernatant cells were washed off the stromal layer; the adherent cell layer was then treated with trypsin and recultured for analysis of collagen biosynthesis, factor VIII associated antigen, and fluorescent Y body determination [3]. Y body analyses were performed on coded specimens which included normal male and normal female controls. The normal male stromal layers had $66 \pm 18\%$ Y bodies ($n = 6$), and the normal female had $1 \pm 1\%$ Y bodies ($n = 3$). At least 200 cells were scored for each sample.

The results of this study demonstrated that the marrow stromal cells became increasingly donor derived with time after transplantation. In five of the six informative cases, that is, male patients beyond 40 days after transplantation with female donors, the percentage of Y bodies seen was $\leqq 5$. In three cases, no Y bodies were seen, and in one case, two were seen in 200 cells. Thus, the data indicated that the cells comprising the in vitro microenvironment following marrow transplantation for leukemia become entirely donor derived. In the two patients with aplastic anemia who did not receive total body radiation in this series, one was female and studied at day

* Supported by Grants CA 16448 and CA 18029 from the National Cancer Institute of the National Institutes of Health and by research funds of the Veterans Administration. A. K. was supported by a Grant from the Medical Research Council of Canada

362 posttransplant. Forty-eight percent Y body positive cells were seen in the stromal layer. Since normal male stromal layers had 66 ± 18% positive cells, 48% is within 1 SD of the male control and thus could indicate that up to 100% of the stromal cells were donor derived.

The observation that MSC were transplantable led us to attempt further characterization. Histochemical stains showed that more than 90% of the explanted stromal cells were peroxidase negative and nonspecific esterase negative. In addition, relatively few cells fluoresced when examined with a monoclonal antibody directed against monocyte-macrophages, the 5F-1. Thus, it appeared that the marrow stromal cells were composed predominantly of cells not in the granulocytic or monocyte-macrophage lineages. An affinity purified antibody directed against factor VIII associated antigen reacted with 5%–25% of stromal cells [4]. Collagen biosynthetic studies were performed by radiolabeling untreated and recultured stromal cells with tritiated proline and tritiated glycine in the presence of beta-aminoproprionitrile and sodium ascorbate followed by polyacrilamide gel electrophoresis after digestion with purified bacterial collagenase and pepsin. The active synthesis of collagen types I, III, and IV was demonstrated [5]. Type IV collagen is synthesized by smooth muscle cells, endothelial cells, and some epithelial cells, but not by fibroblasts. The presence of factor VIII associated antigen and type IV collagen synthesis was demonstrated in stromal cell layers from transplant patients that were close to 100% donor derived. In summary, the studies on MSC following marrow transplantation demonstrated that these cells were transplantable, had certain characteristics associated with endothelial cells such as type IV collagen synthesis and the presence of factor VIII associated antigen, and suggested that there might be a common stem cell for hematopoiesis and its vitro microenvironment.

C. Studies in Chronic Myelogenous Leukemia

The origin and certain features of the cells forming the in vitro microenvironment were studied in a long-term marrow culture derived from a patient with Philadelphia chromosome (Ph′) positive chronic myelogenous leukemia (CML) who was heterozygous for glucose-6-phosphate dehydrogenase (G6PD). Such studies are possible in patients who are heterozygous for the X-linked enzyme G6PD since only one of two X chromosomes is active in each XX somatic cell. Thus, women who are heterozygous for the usual G6PD gene (Gd^B) and a variant such as Gd^A have two cell populations: one synthesizing type A G6PD and the other, B-type enzyme. Skin fibroblasts cultured from Gd^B/Gd^A heterozygotes with CML show both B and A activities, whereas granulocytes, platelets, red cells, monocytes, and some lymphocytes have only a single enzyme type [6]. These data have been interpreted to indicate that CML is a clonal neoplasm arising in a pluripotent stem cell.

The present patient was a 31-year-old nurse who was found to have Ph′-positive CML 6 years prior to study. She had been treated intermittently with busulfan since diagnosis and was in an early accelerated phase when first studied. At that time she had splenomegaly and night sweats. The blood counts included a white blood cell count of $71.1 \times 10^3/mm^3$; platelet count $292 \times 10^3/mm^3$; and hemoglobin 13.4 g/100 ml. The white blood cell differential included 2% blasts and 5% promyelocytes. The marrow showed fewer than 5% blasts in a hypercellular marrow without fibrosis. G6PD studies showed that the red cells, platelets, and granulocytes were type B. Skin, marrow fibroblasts, and T-lymphocytes had equal amounts of A and B activities. Granulocytic colonies and erythroid bursts grown from peripheral blood and marrow were all type B.

A long-term marrow culture was established from the patient and sequentially ended at 3, 6, and 9 weeks for studies of G6PD, collagen biosynthesis, and factor VIII associated antigen. Neither this patient nor six other patients with chronic phase CML produced CFU-C from long-term cultures beyond 4 weeks. This is in striking contrast to cultures from normal individuals which sustain hematopoiesis in excess of 10 weeks in almost all instances. A possible explanation for this phenom-

enon came from an examination of the stromal cell layer. At 3 weeks, the stromal cells had only type B G6PD. However, the stromal cells were actively synthesizing types I, III, and IV collagen, and between 5% and 25% contained factor VIII associated antigen. At 6 weeks, when hematopoiesis had ceased, factor VIII associated antigen positive cells were no longer seen, and some type A G6PD was found in the stromal cell layer. At 9 weeks, equal activities of A and B G6PD were detected, and only types I and III collagen were synthesized. We interpret these data to indicate that at 3 weeks the MSC were synthesizing collagen, were factor VIII positive, and were derived from the leukemic progenitor. This strongly suggests that there is a stem cell common to hematopoiesis and in vitro MSC. The data at 6 and 9 weeks indicated that fibroblasts rapidly became the dominant cell type in the stromal cell layer. In normal stromal cell layers, type IV collagen and factor VIII positive cells have been observed as late as 13 weeks. Perhaps stromal cells derived from the CML clonal progenitor are defective in vitro and thus unable to regulate the outgrowth of fibroblasts. Previous data have demonstrated that marrow fibroblasts in patients with CML do not arise from the neoplastic clone. This in vitro phenomenon may have an in vivo counterpart in the high frequency of development of myelofibrosis in this disorder.

D. Characteristics of Marrow Stromal Cell Precursors

Almost all committed granulocytic and erythroid progenitors, CFU-C, and the BFU-E, express Ia antigen on their cell surface. Between 90% and 100% are lysed when exposed to a monoclonal antibody directed against the Ia determinant (7.2) and complement [7]. In the mouse, it has been shown that most multipotent stem cells (CFU-S) are Ia negative [8]. We examined the ability of an anti-Ia antibody plus complement-treated marrow specimen to form a long-term marrow culture and to generate CFU-C. One such experiment is shown in Fig. 1. In this experiment, the 7.2 antibody plus complement was cytotoxic to 92% of the CFU-C. However, when the treated marrow specimen was used to generate a long-term marrow culture, a morphologically normal MSC layer was formed and CFU-C regenerated. Over the 10-week life of the culture, the cumulative CFU-C yield was equal to 40% of the complement control. However, despite the fact that the complement was not cytotoxic to CFU-C, the complement-treated culture produced significantly fewer CFU-C than did the untreated control. Nevertheless, this experiment demonstrates the ability of an anti-Ia antibody plus complement-treated marrow to regenerate committed progenitors for over 10 weeks and suggests that the stem cells for both MSC and CFU-C

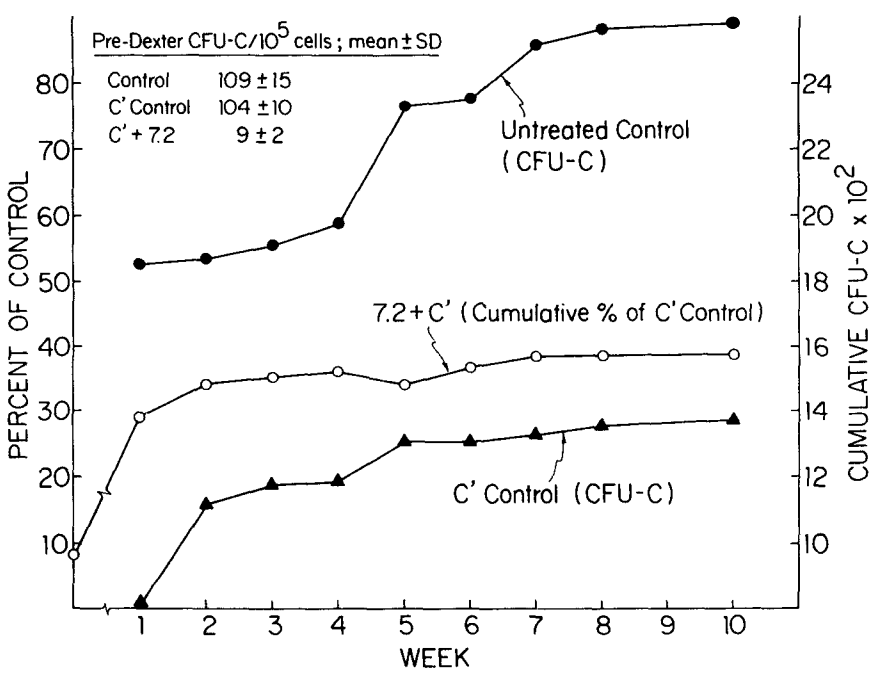

Fig. 1. Normal bone marrow buffy coat cells were treated with a monoclonal antibody directed against Ia-like determinants (7.2) with and without the addition of rabbit complement and then established in long-term cultures. Ninety-two percent of CFU-C were lysed by the antibody with complement; none were lost with the complement control. All cultures generated CFU-C for 10+ weeks. The 7.2 plus complement treated culture generated approximately 40% of the control number of CFU-C over the life of the culture

are Ia negative. Thus, the long-term marrow culture system may be a useful indirect tool for assessing the characteristics of an earlier precursor than those capable of forming colonies in semisolid media.

We found that marrow stromal cells from long-term cultures showed faint fluorescence with two monoclonal antibodies directed against common acute lymphoblastic leukemia antigen (CALLA), the J-5 and the 24.1 ([9], Martin P., personal communication). Studies have shown that both antibodies recognize the same epitope in cross-blocking experiments (Martin P., personal communication). We were also able to demonstrate that the 24.1 plus complement is cytolytic for mature MSC. To determine whether the precursor for MSC also expressed CALLA, we exposed a normal marrow to 24.1 plus complement and then initiated a long-term culture. No differences were noted in the number of CFU-C generated between the complement control and the complement plus antibody-treated specimen. Moreover, normal stromal cell layers developed in both and were subsequently shown to be CALLA positive. This experiment suggested that the precursor cell for marrow stromal cells is CALLA negative and that CALLA arises as a differentiation antigen in MSC.

E. Summary

In a preliminary manner, the data presented here characterize some features of MSC and their progenitors. The progenitors, at least in chronic myelogenous leukemia, are derived from the neoplastic pluripotent stem cell that also differentiates along lymphoid and myeloid pathways. In addition, we have demonstrated that the precursor for MSC is lacking both the Ia and CALLA determinants.

Several antigenic and functional characteristics of the mature stromal cell population have also been identified. Stromal cells express CALLA, synthesize types I, III, and IV collagen, and may express factor VIII associated antigen. It is of interest that fibroblasts do not express factor VIII associated antigen, do not synthesize type IV collagen in measurable quantities, but do express CALLA [9]. Endothelial cells express factor VIII associated antigen, synthesize type IV collagen, but are not CALLA positive. Thus, MSC have some features in common with fibroblasts and others with endothelial cells. The unique characteristics of MSC are that they are transplantable and are derived from a common progenitor with other hematopoietic cells. These features clearly distinguish this cell population from fibroblasts, which are neither transplantable nor derived from the neoplastic clone in CML.

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