METHODOLOGY OF LONG-TERM CULTURE OF HUMAN HEMOPOIEtic CELLS

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SUMMARY: Long-term culture (LTC) of hemopoietic cells has become a relatively specific operational term that is used to refer to a system in which very primitive hemopoietic cells can survive, proliferate, and differentiate into precursors of many lineages in the absence of exogenously added growth factors, but in the presence of other "stromal" cells. The latter are also of marrow origin but are developmentally unrelated to hemopoietic cells. Not surprisingly, therefore, a knowledge of the essential components of the system is required to initiate and maintain cultures in which sustained hemopoiesis will be reproducibly obtained. A considerable body of empirical data has been accumulated to define procedures that achieve this with normal human marrow, and analysis of the system itself has provided an understanding of some of the cellular and molecular dynamics that take place. This review surveys some of the more important features of LTC of human hemopoietic cells, provides associated methodologic information, and summarizes some current and anticipated applications.

Key words: human long-term marrow culture; hemopoiesis; stem cells.

I. INTRODUCTION

In the normal adult, blood cell numbers are maintained by an orderly sequence of proliferation and differentiation events that cells restricted to hemopoiesis undergo in the bone marrow. To characterize the various participating cell populations and to identify important molecular signaling events and gene programming changes, there has been great interest in the development of culture systems that reproduce all aspects of marrow function.

Between 1965 and the late 1970s, the era of in vitro colony assay development had its heyday. As a result, cells from every hemopoietic lineage of either murine or human origin can now be quantitated in semisolid culture systems containing appropriate nutrients and growth factors (30). Indeed, the use of such systems was key to the original discovery and characterization of many of the so-called "hemopoietic growth factors," some of which have kept their initial designation as colony-stimulating factors (7). The purification and characterization of distinct classes of hemopoietic progenitors detected by their ability to produce different types and numbers of clonal progeny in vitro has also played an important role in defining the extensive hierarchical nature of the hemopoietic cell system (12).

Among the most primitive clonogenic cells detectable in both murine and human marrow are progenitors with extensive proliferative capacity (>20 cell divisions), pluripotent myelopoietic potential (>2 lineages), and some self-renewal ability (generation of pluripotent, high proliferative potential progeny detectable in replating experiments) (19,29). In the mouse, primitive hemopoietic cells with these properties can also give rise to cells that home to the spleen and produce macroscopically visible colonies within 9 d (27,31). However, sustained production or self-renewal of any of these clonogenic cells within standard semisolid culture systems is not yet possible, suggesting that other factors or molecular interactions not yet recognized may be required. Moreover, in general the clonal growth of primary hemopoietic cells in semisolid culture systems depends on their continuous stimulation by soluble growth factors that are incorporated into the medium when the culture is initially set up. These culture systems are thus not readily suited to analyzing changing signals from regulatory cells, especially those that may require direct cell-to-cell or cell-to-matrix interactions. Rather, the power of hemopoietic colony assay techniques lies in their use for measuring changes in the number or growth factor responsiveness or requirements of relatively late classes of progenitors, and for investigating differential effects on progenitors at different stages of differentiation or on different hemopoietic pathways or both.

The long-term culture (LTC) system, developed first for murine marrow in the late 1970s (11,24) and successfully adapted for human cells a few years later (8,22,23), offers a complementary approach to the investigation of hemopoiesis because it seems to reestablish in vitro the essential cell types and mechanisms responsible for the localized and sustained production of hemopoietic cells in the marrow in vivo.
Initial studies revealed that the continued production of myeloid cells including clonogenic myeloid progenitors could be readily achieved when marrow was placed in liquid culture at relatively (by comparison to clonogenic assays) high cell densities (>10⁶ cells/ml), providing the appropriate supplements, temperature and feeding procedures were used (Fig. 1). Subsequent analysis of this system has shown why most of these empirically discovered requirements are important and how the system itself can be exploited, not only to analyze discrete regulatory processes but also to provide quantitative assays for very primitive hemopoietic cells not currently measurable by in vitro colony assays. Progress along these lines has been particularly rewarding for the investigation of human hemopoiesis where reliance on culture systems has been paramount.

II. KEY PRINCIPLES

It is now clear that an understanding of the unique features of the LTC system requires an appreciation of the following three basic principles: The appropriate use of time to relate mature (recognizable) cell output to the previous presence of a more primitive cell, the requirement for competent regulatory (stromal) cells as well as for hemopoietic precursors, and the function of regular feeding to activate the system.

A. Time

Because of the hierarchical structure of the hemopoietic system involving many sequential differentiative divisions on each pathway, elapsed time allows the progeny of successively more and more primitive hemopoietic cells present in the initial population seeded into the culture to be detected. The longer the interval since the start of the culture, the more primitive the cell responsible for the output of the mature cells present later at the time of assessment. Thus, if the objective is to use the system to detect the most primitive hemopoietic cell types, the interval between initiation of the culture and its assessment must be sufficient to ensure that the contribution of mature cells from intermediate progenitors is no longer significant. In the case of human marrow, we have found that a 5- to 8-wk time period between initiating cultures and assessing clonogenic progenitor numbers allows quantitation of a very primitive cell in the starting population that seems to be distinct from and precursor to most, if not all, cells detectable in direct clonogenic assays (20,35). We have therefore proposed the operational term of "long-term culture-initiating cell" (LTC-IC) for this cell. The choice of a minimum interval of 5 wk is clearly somewhat arbitrary, but was based on earlier data indicating that 4-hydroperoxycyclophosphamide-resistant hemopoietic cells required at least 4 wk to generate significant numbers of clonogenic cells in this system (>50% of control values), and conversely, that purified clonogenic cells required at least 4 wk to decline to low levels (<10% of input values) in such cultures (35,37).

B. Two components

The second principle concerns the two-component nature of this system. To obtain sustained hemopoiesis, not only is the initial addition of primitive hemopoietic cells required, but an adequate layer of "stromal" cells that provide essential, albeit poorly understood, supportive and regulatory functions must also be present (or be allowed to develop sufficiently rapidly). If either one of these components is omitted, or contact between them prevented, sustained hemopoiesis is not observed (2,13,35). Although both types of cells (i.e. hemopoietic precursors and stromal cells) are present in bone marrow, they are ontologically unrelated and can be physically separated or differentially inactivated (3,14,17,35). Accordingly, provision of one allows independent investigation, even quantitation, of the other (as for example, in the assay for LTC-IC where preestablished irradiated marrow adherent layers are used). Conversely, failure to control for one can confound assessment of the other. Thus for example, the apparent need to initiate cultures with a single inoculum of marrow cells at high density or with sequential inocula can now be recognized simply as procedures that provide adequate numbers of both components within an appropriate time frame.

C. Stimulation not necessarily continuous

The third principle concerns the mechanism that allows continuous hemopoiesis to be maintained in the system. Empirically it was found that weekly half-media changes were convenient and compatible with obtaining sustained hemopoiesis. Subsequent investigation showed that this resulted in cyclic oscillations in the proliferative activity of the most primitive hemopoietic cells in the system, and for human marrow cultures this regulatable behavior is confined to primitive hemopoietic cells that are localized within the adherent layer (4). Because of the close analogy between the hemopoietic cell types responsive to this cycling control in vitro and in vivo, and the involvement of cells related to those that make up the marrow microenvironment, the LTC system also offers a unique approach to the analysis of the molecular basis of how the turnover of

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**Fig. 1.** Diagram summarizing the make-up of LTC initiated with normal human marrow as outlined. Most of the cells are contained within a complex "adherent layer" that forms on the surface of the culture vessel and contains both mesenchymal ("stromal") cells (showing phenotypic characteristics of fibroblasts, adipocytes, endothelial cells and smooth muscle cells) and hemopoietic cells (primarily primitive cells and macrophages). Extending from the adherent layer is a transparent viscous component that contains many entrapped but otherwise free-floating cells (dotted line) and above this viscous layer are truly nonadherent cells (consisting almost exclusively of granulocytes and macrophages after 3 wks). These nonadherent cells are produced continuously from more primitive cells whose support and turnover is dependent on and regulated by interactions with other cells in the adherent layer.