CONCISE REPORT

Demonstration of Burst-Promoting Activity of Recombinant Human GM-CSF on Circulating Erythroid Progenitors Using an Assay Involving the Delayed Addition of Erythropoietin

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We demonstrate through the use of an in vitro assay involving the delayed addition of erythropoietin that human recombinant GM-CSF, cloned from a mature T cell line, Mo, clearly has burst-promoting activity (BPA) on peripheral blood erythroid progenitors at picomolar concentrations. Delay for up to 72 hours of the addition of erythropoietin to semi-solid methylcellulose cultures of concentrated peripheral blood progenitors minimizes or eliminates BPA-independent erythroid colony formation with little loss of BPA-dependent erythroid colony formation. This assay will prove useful in accurately detecting sources of BPA.

ICSOVE PROPOSED in an early study that a single factor could be involved in stimulating the proliferation of early murine hematopoietic progenitors to give rise to multiple cell lineages. One such factor with a wide spectrum of activities, murine interleukin-3 (IL-3), has since been purified and cloned. No human DNA sequence homologous to IL-3 has, however, been reported to date. With the demonstration by Metcalf and his coworkers that purified murine GM-CSF could promote up to five divisions of the early progenitors that give rise to erythroid, eosinophilic, and megakaryocytic colonies, we decided to determine whether or not the recently cloned human GM-CSF could also stimulate the proliferation of circulating erythroid progenitors. Our previous assays of conditioned media for the presence of BPA have been weakened by a relatively high number of BPA-independent BFU-E derived colonies that appear when erythropoietin is added immediately to in vitro cultures. In the past it has been demonstrated that delay of the addition of erythropoietin to cultures reduces the survival of mature erythroid progenitors and encourages the identity of CFU-GEMM derived colonies. Recently a two stage cell culture assay was described using delayed addition of erythropoietin to detect human BPA. Here we report that delayed addition of erythropoietin to methylcellulose cultures of concentrated peripheral blood progenitors improves discrimination between conditioned medium-containing BPA and conditioned medium that does not and demonstrate as well that recombinant GM-CSF has burst-promoting activity on circulating peripheral blood erythroid progenitors.

MATERIALS AND METHODS

Peripheral blood was obtained from normal volunteers and resuspended 1:1 with IMDM in IScove's modified Dulbecco's medium supplemented with 20% fetal calf serum (FCS). The mononuclear cell layer was collected following density centrifugation at 20 °C using a Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient (specific gravity 1.077). The mononuclear cells were washed three times with IMDM + 20% FCS and resuspended at a concentration of 5 x 10^6 cells/mL. These mononuclear cells were then depleted of adherent cells by incubating them on 100 x 15 mm Lux tissue culture dishes (Miles Laboratories, Inc, Naperville, Ill) pretreated with 1mg/mL rabbit anti-mouse IgG + IgA + IgM (H&L) (Zymed Laboratories, Inc, S San Francisco, Calif) for one hour at 4°C, washed twice in IMDM and once in PBS. The cells were then resuspended in PBS + 5% FCS at a concentration of 10 x 10^6 cells/mL and incubated on 100 x 15 mm Lux tissue culture dishes (Miles Laboratories, Inc, Naperville, Ill) pretreated with 1 mg/mL rabbit anti-mouse IgG + IgA + IgM (H&L) (Zymed Laboratories, Inc, S San Francisco, Calif) for one hour at 4°C. Indirect immunofluorescence demonstrated that 4% of the nonadherent cells (routinely 98% viable by trypan blue exclusion) expressed Leu 1, 1% Leu 5, 5% Leu M1, and 4% Leu M2. The nonadherent cells were gently collected and resuspended at a concentration of 5 x 10^6 cells/mL in 0.9% methylcellulose in IMDM with 30% FCS, 0.9% deionized bovine serum albumin (Sigma Fraction V), and 10^-3 M 2-mercaptoethanol. One unit of erythropoietin (Terry Fox Laboratories, Vancouver, BC) or recombinant erythropoietin (Genetics Institute, Cambridge, Mass) in NCTC 109 medium (Whittaker M.A. Bioproducts, Walkersville, Md) or the medium alone was added dropwise to each 1 mL culture at varying time intervals. Colony number was determined 13 to 14 days after the initiation of the culture. Conditioned serum-free medium from the human mature T cell line, Mo, was kindly supplied to us by Dr J. Gasson and Dr D. Golde (University of California, Los Angeles). Recombinant human GM-CSF was cloned from the Mo cell line, and purified from transfected COS-1 cells, as described elsewhere. The same GM-CSF was expressed in E coli and purified by standard methods to be published elsewhere. The yeast-derived recombinant GM-CSF was isolated from the supernatant obtained from cultures of yeast engineered to secrete the hematopoietin directly into the medium. This protein was used in the in vitro study.

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RESULTS

Both Mo\(^{13}\) and MLA-144\(^{2}\)-conditioned medium are known to be potent sources of burst-promoting activity (BPA), however, even in the absence of a source of BPA, there is a relatively high level of BFU-E-derived colony formation when erythropoietin is added immediately to the cultures. By delaying the addition of erythropoietin up to 72 hours, there is a decline in BPA-independent BFU-E-derived colony formation without a loss of BPA-dependent colonies (Fig 1). By delaying the addition of erythropoietin past three days, however, there is a loss of detectable erythroid colonies. Thus, at a cell concentration of \(5 \times 10^5/\text{mL}\), a three-day delay of the addition of erythropoietin appears optimal in limiting the number of BPA-independent BFU-E-derived colonies without significantly reducing the total number of erythroid progenitors.

Using this delayed erythropoietin assay, purified recombinant GM-CSF derived from a variety of expression systems was tested for burst-promoting activity. Figure 2 demonstrates that recombinant human GM-CSF had demonstrable burst-promoting activity while media alone, and conditioned media from mock transfected COS-1, yeast, and \(E\). coli did not. Both the COS-1 (\(-50 \mu\text{g/mL}\)) and yeast (\(-100 \mu\text{g/mL}\)) derived recombinant GM-CSFs continued to be active at a 1:10\(^3\) dilution, equivalent to a protein concentration of 20 and 40 pmol/L, respectively. The \(E\). coli GM-CSF (\(-50 \mu\text{g/mL}\)) was active at a 1:10\(^2\) dilution, equivalent to a protein concentration of 35 nmol/L. Both human urinary and recombinant erythropoietin (data not shown) were equally effective.

DISCUSSION

An assay based upon the delayed addition of erythropoietin that minimizes or eliminates BPA-independent erythroid colony formation has been presented. Using this assay we demonstrate that recombinant human GM-CSF has significant burst-promoting activity on circulating human erythroid progenitors, and that this activity is present at picomolar concentrations of the hematopoietin. It was interesting to note that even the unglycosylated recombinant human GM-CSF derived from \(E\). coli continues to have burst-promoting activity. These findings suggest that besides having the capability of functionally activating granulocytes\(^{14,15}\) and stimulating the proliferation and differentiation of granulocyte/macrophage progenitors,\(^{14}\) recombinant GM-CSF is also capable of stimulating the formation of erythroid colonies in vitro.

Figure 3 is an interpretation of the assay in relation to the capacity of erythroid progenitors to proliferate and differentiate in the presence or absence of BPA and erythropoietin. With the immediate addition of erythropoietin to the methylcellulose cultures, mature progenitors that require only erythropoietin for terminal differentiation are capable of proliferating and terminally differentiating to form BPA-independent BFU-E. Delay of the addition of erythropoietin to the cultures for three days inhibits the survival or function
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... of these erythropoietin-dependent progenitors. On the other hand, if a source of BPA is present, nearly all of these BFU-E remain capable of proliferation. Following the delayed addition of erythropoietin they terminally differentiate into erythrocyte colonies. Thus the signal to noise ratio increases when the addition of erythropoietin is delayed.

This assay which limits BPA-independent growth is useful as a sensitive means of screening proteins for burst-promoting activity. In the present context, we have demonstrated that human recombinant GM-CSF stimulates not only the proliferation and differentiation of granulocyte/macrophage progenitors, but also allows the early erythroid progenitor to survive, grow, and eventually differentiate with the delayed addition of erythropoietin. The wide spectrum of actions of this glycoprotein and the implications of these functions can now be explored.

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