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Recombinant Murine Steel Factor Stimulates In Vitro Production of Granulocyte–Macrophage Progenitor Cells

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Abstract   The ability of murine Steel factor to promote the in vitro production of granulocyte–macrophage progenitor cells (CFU-GM) was examined in short-term liquid cultures. Bone marrow from C57BL/6J or Sl/Sl^d mice was placed in culture for seven days with either Steel factor alone or in the presence of IL-3. CFU-GM responsive to GM-CSF, IL-3, and CSF-1 were measured in the input population and again after 3 or 7 days in culture. Steel factor alone increased the number of all CFU-GM types as early as 3 days after culture initiation, with further increases at day 7. This effect was potentiated by the addition of IL-3. Production of CFU-GM by C57BL/6J or Sl/Sl^d marrow was comparable except for enhanced production of CSF-1 responsive progenitors by Sl/Sl^d marrow. A recombinant Sl^d protein was also shown to be equivalent to the wild-type protein in its capacity to promote CFU-GM production from normal bone marrow.   © 1992 Wiley-Liss, Inc.

Key words: colony forming cells, differentiation, IL-3, CSF1

INTRODUCTION

The gene product of the murine Steel (*Sl*) locus has recently been identified and has been referred to as either mast cell growth factor (MGF) [Anderson et al., 1990; Copeland et al., 1990; Williams et al., 1990], stem cell factor (SCF) [Zsebo et al., 1990a,b], or *kit* ligand (KL) [Huang et al., 1990], referred to here as Sl factor. Sl factor cDNAs encode a protein of 248 amino acids (AA) [Anderson et al., 1990; Huang et al., 1990] with extracellular (185 AA), transmembrane (27 AA), and intracellular domains (36 AA). The Sl^d allele encodes a mutant Sl factor protein that lacks the transmembrane and cytoplasmic domains, resulting in a soluble product that is not membrane bound [Brannan et al., 1991; Flanagan et al., 1991]. The profound hematologic, pigmentation, and germ cell defects in the Sl^d mouse suggest that soluble Sl factor is sufficient for a viable but not a normal phenotype.

Wild-type (+/+) Sl factor has been shown to effect the growth of cells at various stages within the hematopoietic stem and progenitor cell hierarchy. It synergizes with erythropoietin (Epo) to enhance erythroid burst (BFU-E) and mixed colony (CFU-GEMM) growth [Anderson et al., 1990; Broxmeyer et al., 1991a,b; Zsebo et al., 1990b], as well as with a number of growth factors active on granulocyte–macrophage progenitors (CFU-GM) [Broxmeyer et al., 1991a,b], megakaryocyte progenitors (CFU-Mk) [Briddell et al., 1991], bursts (BFU-Mk) [Briddell et al., 1991], and blast cell colony-forming cells (CFU-BL) [Briddell et al., 1991]. Sl factor has a modest stimulatory effect on purified day 14 spleen colony-forming cell (CFU-S) proliferation [de Vries et al., 1991], which can be greatly enhanced in the presence of IL-1, IL-6, and IL-3.

The differentiation of primitive hematopoietic precursor cells can be monitored by the production of in vitro clonogenic cells in short-term liquid cultures [Moore et al., 1987]. In the present studies, we examined the effects of murine Sl factor in this system known as the delta assay using normal C57BL/6 or mutant Sl/Sl^d bone marrow as target cells. The data demonstrated that Sl factor was a potent producer of

clonogenic cells in liquid culture and further demonstrated the equivalent bioactivity of wild type and Sl^d versions of the Sl factor protein in vitro.

MATERIALS AND METHODS

Mice

C57Bl/6J (B6) and Sl/Sl^d mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen free conditions.

Cytokines

Recombinant murine GM-CSF, IL-3, and Sl factor were expressed in yeast as described [Anderson et al., 1990; Park et al., 1986a,b] and purified to homogeneity. The production of recombinant human CSF-1 has also been described [Cerretti et al., 1988]. Two different forms of Sl factor were produced. The wild-type Sl factor encoded amino acids 1–185 of the extracellular domain [Anderson et al., 1990] and the Dickie allele of Steel (Sl^d), which was identical to wild type through amino acids 1–180, but with three unique C-terminal amino acids (Val, Ser, Leu), giving a protein of 183 amino acids [Brannan et al., 1991; Flanagan et al., 1991]. This corresponds to the sequence of the naturally occurring soluble Sl^d molecule [Brannan et al., 1991; Flanagan et al., 1991].

Colony Assays

Agar colony assays were carried out as previously described [Williams et al., 1987]. Briefly, bone marrow was seeded into 0.5 ml wells in 0.3% agar with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), McCoy's Medium (Gibco, Grand Island, NY) and the appropriate growth factor (100 ng/ml final concentration). Quadruplicate cultures were initiated in each experimental group and colonies of ≥ 40 cells were scored with an inverted microscope after 7 days of incubation in a humidified atmosphere of 7% O_2, 8% CO_2, and 85% N_2.

Liquid Cultures

Bone marrow cells were suspended in McCoy's medium with 10% FBS at a final concentration of 1×10^6 cells per ml. Ten-ml cultures were initiated at time zero and supplemented with 100 ng/ml Sl factor and/or 100 ng/ml IL-3, or no growth factors. Cultures were harvested after 3 or 7 days incubation at 37°C in a fully humidified atmosphere of 6.5% CO_2 in air. Viable cells were counted by trypan blue dye exclusion and cells were analyzed for CFU-GM as described.

RESULTS

Sl Factor Promotes In Vitro Granulocyte–Macrophage Progenitor Cell (CFU-GM) Production From Normal and Sl/Sl^d Bone Marrow

The influence of Sl factor on the survival or production of CFU-GM was assessed in short-term liquid cultures of unfractionated bone marrow cells. The technique employed in these studies was based on the delta assay reported by Moore and Warren [1987]. The absolute number of CFU-GM was determined at time zero by culture in soft agar in the presence of saturating concentrations of either IL-3, GM-CSF, or CSF-1. An identical aliquot of these bone marrow cells was then cultured in medium alone, IL-3 (100 ng/ml), Sl factor (100 ng/ml), or Sl factor plus IL-3 for 3 or 7 days, and the absolute number of CFU-GM responding to GM-CSF, IL-3, and CSF-1 was determined.

The input number of CFU-GM responsive to IL-3, GM-CSF, and CSF-1 in normal B6 or Sl/Sl^d bone marrow was comparable (Fig. 1). Liquid cultures initiated in the absence of any exogenous growth factor led to a steady decline in the frequency of all three classes of CFU-GM, in agreement with previous studies [Williams et al., 1985] from cultures of B6 or Sl/Sl^d bone marrow (Fig. 1). Liquid cultures initiated in the presence of IL-3 alone showed enhanced recovery of CFU-GM responsive to IL-3, GM-CSF, or CSF-1 above that observed with media control; however, no evidence for production of CFU-GM was observed with either B6 or Sl/Sl^d bone marrow in three experiments (Fig. 1). The kinetics of CFU-GM loss in IL-3 containing liquid cultures of B6 and Sl/Sl^d marrow was not significantly different (Fig. 1). Liquid cultures containing Sl factor alone or in combination with IL-3 showed significant increases in the absolute number of CFU-GM responsive to GM-CSF, IL-3, or CSF-1 with both B6 and Sl/Sl^d marrow (Fig. 1). CFU-GM production was greatest in liquid cultures containing both IL-3 and Sl factor (Fig. 1). Production of CFU-GM responsive to IL-3 or GM-CSF was not significantly different between BL6 and Sl/Sl^d bone marrow from liquid cultures containing Sl factor or Sl factor plus IL-3. When liquid cultures were initiated
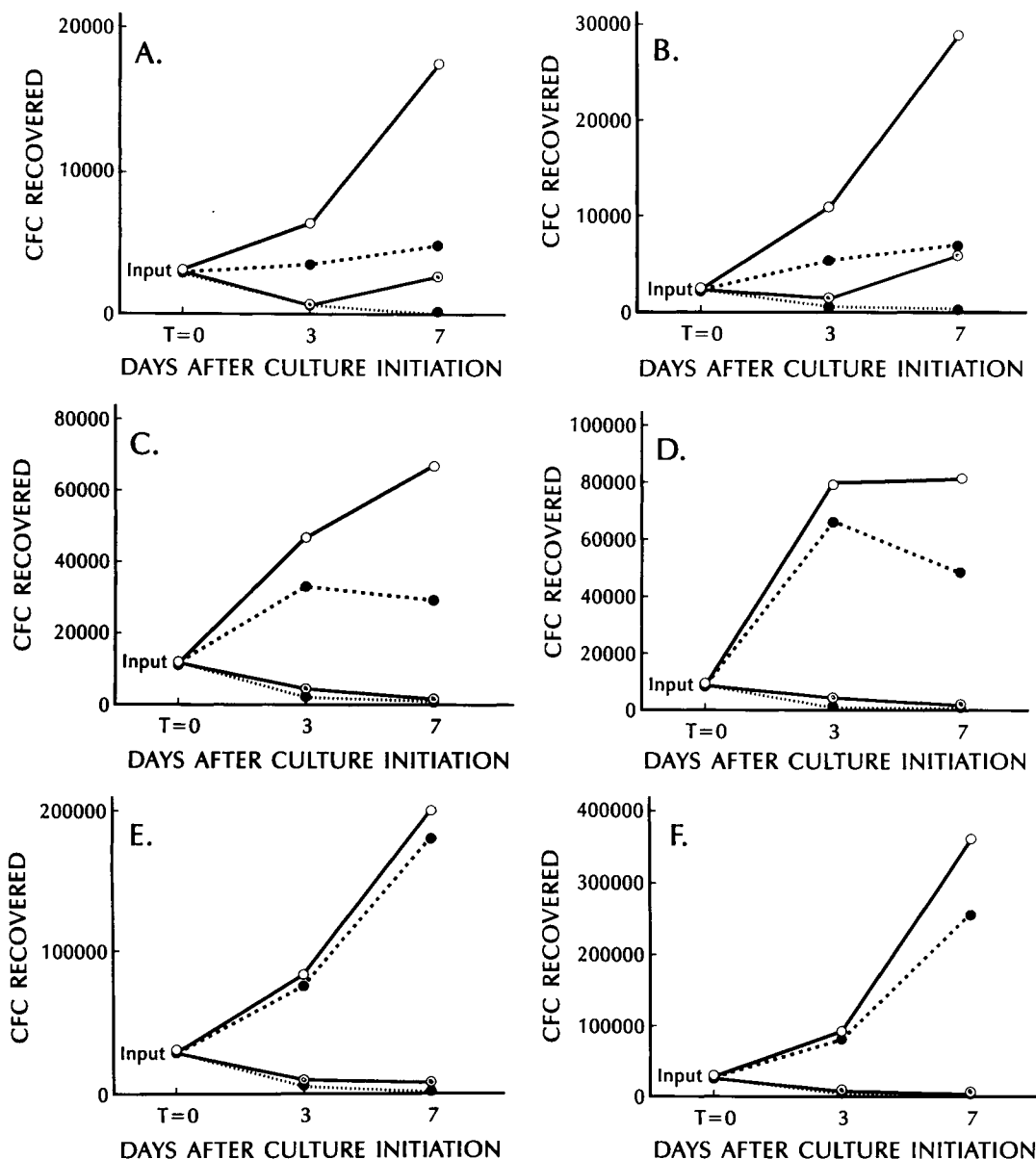
Fig. 1. Absolute colony numbers isolated from liquid cultures of normal C57Bl/6J (**A,C,E**) or *Sl/Sl^d* (**B,D,F**) bone marrow cultures. IL-3-responsive colonies (A,B), GM-CSF-responsive colonies (C,D), and CSF-1-responsive colonies (E,F) were enumerated from 7-day agar cultures as described under Materials and Methods. Liquid cultures were initiated with control medium only (···●···), IL-3 (—◉—), SI factor (—●—) or SI factor plus IL-3 (—○—). Data represent 1 of 3 representative experiments.

with Sl factor plus IL-3, the level of CFU-GM production was 6.9- vs. 8.9-fold for GM-CSF responsive CFU-GM, and 6.7- vs. 5.5-fold for IL-3 responsive CFU-GM with B6 and *Sl/Sl^d* bone marrow, respectively (Fig. 1). By contrast, CFU-GM responsive to CSF-1 were produced at a higher frequency from *Sl/Sl^d* bone marrow than B6 bone marrow in liquid cultures containing Sl factor plus IL-3 (6.7- vs. 14-fold increase at day 7 for B6 and *Sl/Sl^d*, respectively; Fig. 1)

or Sl factor alone (6.7- vs. 10.4-fold with B6 or *Sl/Sl^d* marrow, respectively; Fig. 1).

Wild Type and *Sl^d* SI Factor Have Equivalent Biological Activity In Vitro

The biological activity of the full length 185 amino acid extracellular domain of Sl factor was compared to that of a recombinant version of the *Sl^d* gene product, a naturally occurring soluble form of the growth factor [Brannan et al.,
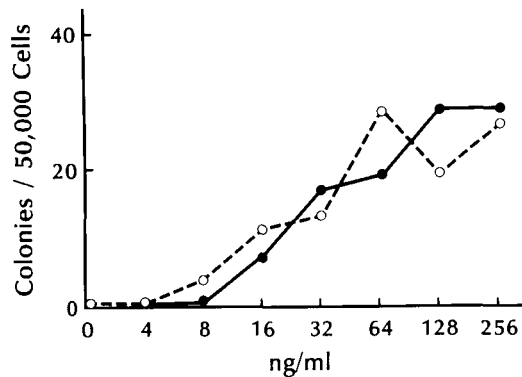
Fig. 2. Comparative titration of wild-type (○) and Sl^d (●) Sl factor on normal murine bone marrow in a 7-day agar culture assay.

Fig. 3. Comparative stimulation of the production of GM-CSF responsive CFC in liquid cultures of normal bone marrow cells stimulated by wild type (—○—) and Sl^d (···●···) Sl factor alone, or wild type plus IL-3 (- - ○ - -) or Sl^d plus IL-3 (- - ● - -). IL-3 alone is shown as (—⊙—). One of two representative studies.

1991; Flanagan et al., 1991]. Biological activity was compared by parallel titration of wild type and Sl^d Sl factor in an agar colony assay using normal B6 bone marrow as a target cell population. Small colonies develop in these cultures after 7 days and contain primarily undifferentiated myeloid cells [Broxmeyer et al., 1991b; Zsebo et al., 1990b]. Figure 2 illustrates that the two forms of Sl factor gave similar titration curves with half-maximal activity in the 10–20 ng/ml range and comparable numbers of colonies stimulated at saturating concentrations.

As an additional means of assessing the comparative bioactivity of the two forms of Sl factor, normal B6 bone marrow was set up in liquid culture as described above with either wild type or Sl^d Sl factor with or without IL-3 to assess the production of CFU-GM. The kinetics and magnitude of CFU-GM production stimulated by both forms of Sl factor were essentially the same (Fig. 3).

DISCUSSION

The production of hematopoietic colony-forming cells in liquid cultures of murine bone marrow has been shown to be enhanced by a number of cytokines [Moore et al., 1987]. The delta assay is thought to measure a differentiation inducing effect on primitive precursors into clonogenic cells with little or no intrinsic self-renewal potential, such as CFU-GM [Williams et al., 1985]. Serial replating studies have documented the inability of CFU-GM to self-renew to any significant extent [Williams et al., 1988]. The current studies examined the ability of Sl factor to promote CFU-GM generation in a delta-type assay.
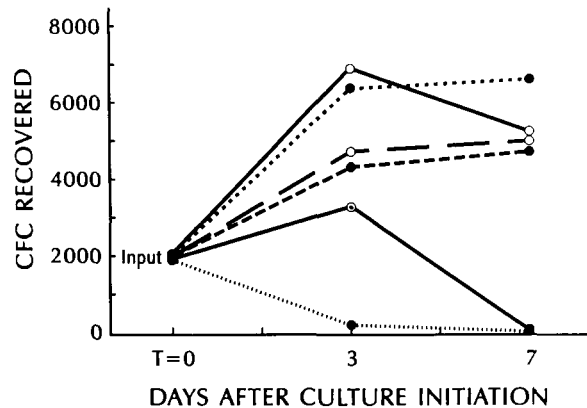
Characterization of the Sl and W defect of mice indicated that the mutations effect cells very near the pluripotent hematopoietic stem cell stage [Russell, 1979]. Previous studies have documented that Sl factor acts on very primitive hematopoietic progenitors such as day 14 CFU-S [de Vries et al., 1991], high proliferative potential progenitor cells (HPP-CFC) [Zsebo et al., 1990b], long-term culture-initiating cells [Brandt et al., 1991], CFU-GEMM [Anderson et al., 1990; Broxmeyer et al., 1991a,b; Zsebo et al., 1990b], and primitive T- and B-cell precursors [Williams DE: unpublished observations; McNiece et al., 1991]. Profound synergy has been reported between IL-3 and Sl factor on a number of these populations [Anderson et al., 1990; Broxmeyer et al., 1991a,b; de Vries et al., 1991; Zsebo et al., 1990b], and we sought to examine the role of these factors alone and in combination on CFU-GM production in vitro. The data indicated that Sl factor is a potent stimulator of CFU-GM production alone, and this action is enhanced by the addition of IL-3. Similar data were obtained with either normal B6 or Sl/Sl^d target marrow cells. One exception was the production of CSF-1 responsive progenitors, which was enhanced in Sl/Sl^d mice. The significance of this is unclear, given the absence of a gross macrophage defect in these mice [Russell, 1979]. The response of Sl/Sl^d marrow in vitro to Sl factor is consistent with in vivo treatment data with this factor showing erythroid as well as granulocyte–macrophage effects [Zsebo et al., 1990a].

The recent observation that the Sl^d allele encodes a soluble Sl factor poses interesting questions about the relevance of soluble versus membrane bound versions of the growth factor [Brannan et al., 1991; Flanagan et al., 1991], both of which are present in wild-type mice. It is clear from the studies reported here that in those assays examined, the Sl^d and wild-type Sl factor proteins have identical biological activities in vitro. This has recently been observed for murine germ cells as well [Dolci et al., 1991]. Previous studies comparing the capacity of wild-type stromal cells with Sl/Sl^d-derived stromal cells to support in vitro mast cell survival also showed that while transient maintenance was observed on the Sl/Sl^d cells beyond that seen with no stroma, the wild-type cell line promoted long-term survival of mast cells [Boswell et al., 1990]. Recent flow cytometric analysis of +/+ and Sl/Sl^d stromal cells with an antibody specific for murine Sl factor documented the presence of membrane bound Sl factor on the +/+ cells, but not the Sl/Sl^d cells (Williams DE, unpublished observations). Thus, despite comparable mRNA levels in +/+ and Sl/Sl^d stroma for Sl factor, a fundamental difference in the capacity to support mast cell [Boswell et al., 1990] and germ cell [Dolci et al., 1991] survival exists and correlates with the presence of the membrane bound form of the factor. It is clear that soluble Sl factor is active in vitro and in vivo; however, the fundamental question of the natural role of the two distinct forms of the molecule remain to be answered.

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