Multi-CSF-Dependent Colony Formation by Cells of a Murine Hemopoietic Cell Line: Specificity and Action of Multi-CSF

By D. Metcalf

Cells of the Multi-CSF (IL-3)-dependent hemopoietic cell line 32D c13 formed colonies of varying size in agar cultures stimulated by Multi-CSF. Colony formation was linear with respect to cultured cell numbers; colony numbers and size increased with increasing concentrations of Multi-CSF, and 32D colonies themselves contained a high frequency of clonogenic cells. Clonogenic 32D cells died in the absence of Multi-CSF (half-life six hours), and most were unable to complete cell cycles in progress at the time of withdrawal of Multi-CSF. The concentration of Multi-CSF directly influenced the length of the cell cycle of dividing 32D cells. Purified GM-CSF, G-CSF, or M-CSF had no capacity to support the survival or proliferation of 32D cells. Colonies formed by 32D cells appear to offer a useful model for analyzing the action of Multi-CSF in controlling self-renewal by clonogenic hemopoietic cells.

© 1985 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Agar Cultures

Cultures were performed in 35-mm Petri dishes containing 1 mL of Dulbecco's modified Eagle's medium in a final concentration of 20% fetal calf serum and 0.3% agar. The cultures contained varying numbers of cells from the continuous line 32D c13 (usually 100 to 300 per culture) or 75,000 C57BL bone marrow cells. Materials containing CSF were added in volumes of 0.1 mL to the empty culture dish before addition of the cell suspension in agar medium, and cultures were incubated for up to seven days in a fully humidified atmosphere of 10% CO₂ in air.

Cell Lines and Other Cells Cultured

The cloned continuous cell line 32D c13, supplied by Dr J. Greenberger (Boston), was maintained in suspension culture in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum containing 0.1 mL of pokeweed mitogen-stimulated spleen cell-conditioned medium (SCM) per milliliter of culture medium. Cells were washed thoroughly by repeated centrifugation to remove residual SCM before culture in agar medium.

The WEHI-3B D⁺ myelomonocytic leukemia was maintained in suspension culture in Petri dishes containing Dulbecco's Modified Eagle's Medium plus 10% fetal calf serum and subpassaged at weekly intervals.

Normal bone marrow and other cells were obtained from 2- to 3-month-old C57BL/6 mice maintained in this institute.

Multi-CSF and Other Colony-Stimulating Factors

Pokeweed mitogen-stimulated BALB/c SCM was prepared as previously described. Purification was completed by two sequential reverse-phase HPLC fractionations, followed by a final size-based HPLC fractionation (R.L. Cutler, D. Metcalf, N.A. Nicola, unpublished observations). Other purified preparations used were GM-CSF (3 × 10⁸ U/mg) purified from mouse lung-conditioned medium, M-CSF (3 × 10⁸ U/mg) purified from L-cell-conditioned medium (kindly supplied by Dr A.W. Burgess), and G-CSF (6 × 10⁸ U/mg) purified from mouse lung-conditioned medium (kindly supplied by Dr N.A. Nicola).

From the Cancer Research Unit, Walter and Eliza Hall Institute, Royal Melbourne Hospital, Victoria, Australia.

Supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria, the National Health and Medical Research Council, Canberra, and the National Institutes of Health Grant No. CA-25972.

Submitted Feb 9, 1984; accepted Aug 1, 1984.

Address reprint requests Dr D. Metcalf, Cancer Research Unit, Walter and Eliza Hall Institute, Royal Melbourne Hospital, P.O. 3050, Victoria, Australia.

© 1985 by Grune & Stratton, Inc.

0006-4971/85/6502-0016$03.00/0

Blood, Vol 65, No 2 (February), 1985: pp 357-362
All purified preparations of Multi-CSF, GM-CSF, M-CSF, and G-CSF formed single bands in silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis preparations, and units of activity were calculated from colony-stimulating activity in cultures of 75,000 C57BL marrow cells, assigning 50 units of activity to the concentration stimulating 50% maximal colony numbers.

**Colony and Clone Scoring**

Colony formation by 32D cells was scored routinely after seven days of incubation using an Olympus (Tokyo) stereo zoom dissection microscope. Discrete cell aggregates of 50 or more cells were scored as colonies. In some studies, all clones of two or more cells were scored at 40x magnifications. When doubt existed as to the cell content of a small clone, the clone was disaggregated using a fine, orally controlled pipette to allow unequivocal cell counts.

**Recloning of Paired Daughter Cells**

Agar cultures of 300 32D cells were initiated using 0.1 mL of a low (1:32) concentration of SCM. At 15 hours, individual daughter cell pairs were removed using a finely drawn, orally controlled glass pipette. Each daughter cell was transferred to the surface of a 1-mL agar-medium culture containing neither cells nor SCM. The paired cells were separated and washed in the film of surface liquid present on agar-medium cultures. One cell was then transferred to a culture dish containing 1 mL agar medium and 0.1 mL SCM 1:1, and the other cell was transferred to a culture dish containing 1 mL agar medium and 0.1 mL SCM 1:8. After incubation intervals of 12, 24, and 48 hours, the transferred cells were reexamined and the number of progeny present recorded. After seven days of incubation, all clones present were removed and stained with orcein for total cell counts.

**Cell Counts**

Counts on cells for culture were made using a hemocytometer and eosin to detect nonviable cells. Mean colony cell counts were performed on pools of colonies resuspended in 1 mL of medium.

**RESULTS**

Preliminary studies on eight factor-dependent continuous cell lines showed that all were capable of forming colonies in agar medium containing 0.1 mL SCM, and the cloned cell line 32D c13 was chosen as a representative example for detailed analysis. The 32D cells were heterogeneous in size (Fig 1A, B). Most contained metachromatic granules of varying size, but granule numbers per cell were variable and usually lower than in normal mast cells. Approximately 5% of the cells had less basophilic cytoplasm and a ring-shaped or deeply indented nucleus. Occasional giant or multinucleate cells were present, usually with metachromatic granules, in the cytoplasm. No cells had a morphological appearance corresponding exactly to any normal hemopoietic cell. Their full differentiation potential remains uncertain, but under the culture conditions used, mast cell differentiation appeared to predominate.

**Colony Formation by 32D Cells**

In cultures of 300 32D cells stimulated for seven days by supramaximal concentrations of SCM, relatively compact spherical colonies of variable size (200 to 20,000 cells) were present (Fig 1C, D). There were few clones of subcolony size and no surviving single cells in the medium between the colonies. The morphology of colony cells was similar to that of 32D cells maintained in suspension cultures. Colony formation was linear with respect to cultured cell numbers (cells cultured, 10 to 400 per culture) in cultures containing a supramaximal concentration of SCM. In 24 separate experiments using 32D cells, the mean cloning efficiency (frequency of colony-forming cells) was 39 ± 12%.

Colony formation by 32D cells exhibited a typical sigmoid dose-response curve of colony formation with increasing SCM concentrations (Fig 2). The 32D cells were less responsive at lower SCM concentrations than normal GM colony-forming cells, but in both types of culture, maximal colony numbers developed at the same SCM concentration. The steeper dose-response curves for 32D cells suggest that, although 32D cells were heterogeneous in responsiveness, they exhibited a more uniform responsiveness than normal marrow colony-forming cells.

In cultures stimulated by Multi-CSF purified to homogeneity from SCM, identical colonies developed with a similar plating efficiency (30% to 40%) to those stimulated by unfractionated SCM, and the same relationship between colony numbers and Multi-CSF
concentration was observed as in cultures stimulated by crude SCM (Fig 3). With increasing concentrations of purified Multi-CSF, mean colony size continued to increase beyond the point where maximal colony numbers had been achieved (Fig 3), a characteristic also seen with CSF-stimulated colony formation by normal marrow cells.

Recloning Capacity of 32D Colony Cells

The 32D colonies were stimulated to develop by high or low SCM concentrations and then at seven days were resuspended and recultured in agar medium containing SCM. As shown in the example in Table 1, 32D colony cells were readily reclonable, the percentage of clonogenic cells being, in general, higher than in primary cultures of suspension-cultured cells.

Effects of Delayed Addition of Multi-CSF on 32D Cells

To examine the response of 32D cells to initial culture in the absence of Multi-CSF, 32D cells were washed twice and then cultured at a concentration of 300 cells per milliliter in agar medium lacking Multi-CSF. At intervals, 0.1 mL of SCM was layered on the surface of these cultures. Normal marrow cells were cultured in parallel. A result typical of four experiments of this type is shown in Fig 4. The 32D cells were consistently more sensitive to culture in the absence of CSF than were normal GM progenitor cells. Normal GM progenitor cells exhibited a survival half-life of 33 hours, whereas the survival half-life for clonogenic 32D cells was only six hours.

Action of Other Granulocyte-Macrophage CSFs

In cultures containing supramaximal concentrations (400 units) of purified GM-CSF, M-CSF, or G-CSF, no colony formation was observed in cultures of 300 32D cells, and at seven days, the cultures contained no surviving cells. In view of the ability of GM-CSF and G-CSF to initiate but not sustain proliferation in a number of hemopoietic lineages (multipotential, erythroid, eosinophil, and megakaryocyte, \textsuperscript{17,20} cultures were initiated with 400 units of GM-CSF, G-CSF, or M-CSF, and at two days, 0.1 mL of SCM was added. No clones were observed at two days and no colonies or surviving cells were observed seven days after the addition of SCM, indicating that none of these CSFs had the capacity to stimulate the initial proliferation of 32D cells or even to support the survival of these cells in culture.

![Graph](https://example.com/graph.png)

**Fig 2.** Increase in colony numbers with increasing SCM concentrations in cultures of 300 32D cells compared with GM colony formation by 75,000 normal mouse bone marrow cells. Maximum colony numbers were 118 ± 10 for the 32D cultures and 125 ± 15 for the bone marrow cultures. Each point represents mean data from quadruplicate cultures; vertical bars indicate the standard deviations of individual colony counts.

**Fig 3.** Colony formation by 300 32D cells stimulated by purified Multi-CSF. Note the same sigmoid dose-response curve as noted in cultures stimulated by crude SCM, and that mean colony size increases progressively with increasing Multi-CSF concentrations. Vertical bars indicate standard deviations of colony counts. Maximum colony numbers per culture were 110 ± 10. Mean colony size determined from pools of 20 sequential colonies.

**Fig 4.** Effects of initial culture of 32D cells or normal marrow cells in cultures lacking SCM. With increasing delays before the addition of SCM, a progressive fall occurs in the number of colonies that are able to develop owing to death of clonogenic cells. The 32D clonogenic cells exhibit a survival half-life of only six hours compared with 33 hours for normal marrow GM progenitor cells. Each point represents the colony number in an individual culture dish.

**Table 1. Recloning Ability of 32D Colony Cells**

<table>
<thead>
<tr>
<th>SCM Concentration in Primary Cultures</th>
<th>Colony Type Recloned</th>
<th>Mean No. of Cells per Colony</th>
<th>Mean No. of Colony-Forming Cells per Colony</th>
<th>Percent Clonogenic Cells in Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>Large</td>
<td>5,230</td>
<td>4,280</td>
<td>82</td>
</tr>
<tr>
<td>1:1</td>
<td>Small</td>
<td>160</td>
<td>120</td>
<td>75</td>
</tr>
<tr>
<td>1:32</td>
<td>Small</td>
<td>420</td>
<td>350</td>
<td>83</td>
</tr>
</tbody>
</table>

Pools of two to 16 seven-day colonies were resuspended in 23 mL of agar medium and then recultured for seven days in the presence of 0.1 mL SCM. Mean data from quadruplicate cultures.
The failure of GM-CSF, G-CSF, and M-CSF to stimulate 32D cells is unlikely to be due to inactivation of these CSFs by products from 32D cells, since these CSFs remain able to stimulate normal colony formation in cocultures of normal and 32D cells and are unable to stimulate the 32D cell proliferation in cultures of single 32D cells. Recent radiolabeling data (A.W. Burgess, F. Walker, N.A. Nicola, unpublished observations, February 1984) indicate that 32D cells lack receptors for G-CSF and probably also for GM-CSF.

Initiation of Proliferation in Cultures of 32D Cells Stimulated by SCM

Normal GM progenitors are characterized by a marked asynchrony in onset of proliferation in CSF-stimulated semisolid cultures, but cells from leukemic cell lines commence proliferation more uniformly and with a much shorter delay. To determine whether Multi-CSF–dependent cell lines resemble normal or leukemic cells in this behavior, the development of clones of two or more cells was followed in cultures of (a) 100 32D cells stimulated by a tenfold excess concentration of SCM, (b) 10,000 C57BL marrow cells with the same stimulus, and (c) 100 WEHI-3B cells in cultures with no added stimulus.

All three cell populations exhibited a lag period before proliferation commenced in vitro (Fig 5). For leukemic WEHI-3B cells, this lag was minimal; by three hours some cells had completed their first division in vitro and by 15 hours all clonogenic cells had undergone the first cell division. In sharp contrast, and in agreement with previously published data, no cell divisions were evident in normal marrow cultures before 14 to 16 hours, and by 30 hours the number of initiated clones was still only 30% of maximum. The 32D cells closely resembled WEHI-3B leukemic cells, except for an initial slower rate of accumulation of daughter cell pairs in the three- to six-hour culture interval. The data offer a novel method for determining mean cell cycle times, and it is likely, in the cultures used, that WEHI-3B and 32D cells both had cell cycle times of approximately 12 hours. In agreement with this interpretation, the first sets of four granddaughter cells of clonogenic 32D cells were observed in 32D cultures at 17 to 19 hours.

The 32D cells cultured without SCM exhibited essentially no cell divisions in the first eight hours, and after this time few living cells were detectable in the cultures. To initiate factor-free cultures of 32D cells requires washing the cells by centrifugation to remove the SCM used in the maintenance cultures. However, uncentrifuged 32D cells showed the same slow kinetics of initiation. Conversely, centrifugation and washing of WEHI-3B cells did not prolong the short initiation period exhibited by these cells. With a survival half-life of six hours in the absence of SCM, approximately half the clonogenic 32D cells would still be alive after eight hours of culture in the absence of SCM. The failure of daughter cell pairs to appear during this interval suggests that, after withdrawal of Multi-CSF, 32D cells are unable to complete cell cycles in progress when the factor-free cultures were initiated.

Direct Effects of Multi-CSF on Cell Cycle Times of 32D Cells

To determine whether Multi-CSF influences the length of the cell cycle in 32D cells and to avoid problems raised by the heterogeneity of these cells, studies were made on the growth rates of paired daughters of clonogenic cells in cultures containing either SCM 1:1 or SCM 1:8. Comments on the data are restricted to those pairs in which both cells survived and at least one cell exhibited evidence of proliferation. After 24 hours of incubation, there were 16 examples in which both transferred daughters had generated the same number of progeny, 20 examples in which the daughter cell had generated more progeny in the 1:1 recipient plate than in the 1:8 recipient plate, and five examples in which the reverse was true. This trend was also evident at the 48-hour posttransfer observation time (Table 2). There were seven daughter pairs that had generated equal numbers of progeny in the two SCM concentrations, 34 examples in which the paired daughter cell had generated more progeny in 1:1 recipient plate than in 1:8 recipient plate, and 12 examples in which the reverse was true.

The pooled data from paired daughter cells in which both daughters exhibited progressive proliferation fitted logarithmic growth curves, the mean doubling times for cells growing in the presence of 1:1 SCM

---

**Fig 5.** Initiation of proliferation of clonogenic cells assessed by whole-culture counts of the total number of daughter cell pairs in cultures of 100 32D cells stimulated by 0.1 ml SCM, 10,000 C57BL marrow cells stimulated by 0.1 ml SCM, or 100 WEHI-3B leukemic cells with no added stimulus. Data from three experiments pooled and replotted as percent maximal clone numbers. Each point represents mean data from four cultures; vertical bars indicate the standard deviations of individual values.
Table 2. Proliferation After 48 Hours of Incubation of Individual Cells From 32D Daughter Pairs Recloned in High (1:1) or Low (1:8) SCM Recipient Cultures

<table>
<thead>
<tr>
<th>Clone Size in 1:8 SCM Recipient Cultures</th>
<th>Clone Size in 1:1 SCM Recipient Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>15 or more</td>
</tr>
</tbody>
</table>

being 11.5 hours and for cells growing in the presence of 1.8 SCM being 15.0 hours. The data for the 1:1 cultures are in good agreement with cell cycle estimates from Fig 5 of 12 hours for 32D cells in 1:1 SCM and indicate that neither the micromanipulation procedure nor the culture of a solitary 32D cell significantly influenced the subsequent proliferative activity of these cells. This was confirmed by the fact that by seven days, individual micromanipulated single cells had generated colonies containing up to 5,000 cells.

Because unfractionated SCM was used in the daughter cell experiments it is formally possible that the accelerated proliferation of cells in high SCM concentrations is not due to Multi-CSF, but to a contaminating enhancing factor. This is unlikely, however, since higher concentrations of purified Multi-CSF stimulate the more rapid growth of all 32D clones (see Fig 3), and since tests have failed to reveal the presence of enhancers of 32D cell proliferation in SCM fractions.

DISCUSSION

These studies have shown that cells of the factor-dependent hemopoietic cell line 32D c13 are able to generate colonies of varying size in semisolid cultures, that colony formation is linear with respect to cultured cell numbers, and that the clonogenic cells self-generate during colony formation. Colony formation was absolutely dependent on Multi-CSF-containing conditioned medium or Multi-CSF purified from this source, and the overall responsiveness of 32D cells was similar to that of normal GM progenitor cells. Evidence for the uniqueness of this responsiveness to Multi-CSF was the demonstration that purified GM-CSF, G-CSF, or M-CSF was unable to support the survival or stimulate the proliferation of 32D cells.

Multi-CSF plays an important role in controlling the proliferation in vitro of normal stem and multipotential hemopoietic cells. These cells have a significant capacity for self-renewal but are difficult to obtain in large numbers or in purified populations. Clonal cultures of Multi-CSF-dependent 32D cells therefore offer a potentially valuable model for analyzing at the clonal level some aspects of the role of Multi-CSF in controlling self-generation by clonogenic hemopoietic cells, despite the need for caution in the use of cells that are likely to exhibit certain intrinsic abnormalities.

The present analysis showed that clonogenic 32D cells died rapidly (half-life six hours) after withdrawal of Multi-CSF and could not complete cell cycles in progress at the time of Multi-CSF withdrawal. Similar effects have previously been reported on normal GM progenitor cells after GM-CSF withdrawal, but the effects are more rapid on 32D cells and have been ascribed to falling intracellular concentrations of adenosine triphosphate. A comparison of the proliferation of separated paired daughter cells of clonogenic 32D cells showed that Multi-CSF concentration had a direct action in influencing cell cycle times, a similar phenomenon to that documented previously for the action of purified GM-CSF on normal GM progenitor cells.

Clonogenic 32D cells resemble normal cells in their dependency on and responsiveness to CSF stimulation
but resemble cells of continuous leukemic cell lines in their ability to initiate clone formation without the prolonged delay shown by normal cells and in their ability to exhibit extensive self-renewal.\textsuperscript{18, 24} Although the present observations show that 32D cells share properties intermediate between normal and leukemic cells, these observations do not necessarily place the continuous cell lines in a direct sequence between normality and leukemia, and their exact status requires further investigation.

REFERENCES

Multi-CSF-dependent colony formation by cells of a murine hemopoietic cell line: specificity and action of multi-CSF

D Metcalf