Differentiation State and Responses to Hematopoietic Growth Factors of Murine Myeloid Cells Transformed by myb

By Thomas J. Gonda, Elizabeth M. Macmillan, Philip V. Townsend, and Andrew J. Hapel

Murine hematopoietic cells can be transformed in vitro by recombinant retroviruses that express the myb oncogene, and hematopoietic growth factor (HGF)-dependent myeloid cell lines can be derived from these transformed primary cells. In this study, the differentiation state and responses of myb-transformed hematopoietic cells (MTHCs) have been investigated. We find that MTHCs exhibit properties of early myeloid progenitors including synergistic responses to combinations of HGFs and expression of certain surface markers. As reported previously, MTHCs respond well to granulocyte-macrophage colony-stimulating factor (GM-CSF) but can also respond to interleukin-3 (IL-3); the response to the latter factor depends on the mouse strain from which the cells are derived. Although these single factors stimulate MTHCs, combinations of these factors with colony-stimulating factor-1 (CSF-1 or M-CSF) or Steel factor (SLF or SCF) act synergistically to promote colony formation. The surface markers expressed by MTHCs include both granulocyte-macrophage lineage specific antigens Gr-1, 7/4, F4/80, and Mac-1, as well as two antigens found on early progenitors and stem cells—Thy-1 and Sca-1 (Ly6E). Expression of the latter markers is often heterogeneous and can be modulated by the growth factors to which the cells are exposed. Finally, we show that monocytic differentiation of MTHCs can be induced by exposure to tumor necrosis factor (TNFα). Taken together, these results suggest that MTHCs will be a useful model for studying HGF/cytokine responses in both proliferation and differentiation.

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clude biochemical dissection of responses—both proliferative and differentiative—to particular HGFs and cytokines and of synergy between HGFs.

MTHCs may also prove useful as a model for myeloid leukemias such as acute myeloid leukemia (AML). Like the latter, they exhibit characteristics of immature myeloid cells that fail to undergo normal differentiation and require HGFs for proliferation in vitro. Although not overtly tumorigenic on transplantation—again a property shared with most primary AML cells—they can become so under certain conditions, such as conversion to HGF-independence or, in at least one case, transplantation into a sublethally irradiated recipient.

As a first step in exploiting this system, we have undertaken a detailed characterisation of the MTHCs with regard to their differentiation state and potential and to their requirements for and responses to HGFs and cytokines. We show here that MTHCs do indeed exhibit many properties of early myeloid progenitors, and that they can undergo cytokine-induced monocytic differentiation. We also demonstrate that MTHCs respond only to certain HGFs or synergistically acting HGF combinations and that they appear to require such combinations for optimal proliferation. These include particular combinations of GM-CSF, colony-stimulating factor-1 (CSF-1 or M-CSF), Steel factor (SLF), also known as mast cell growth factor [MGF], stem cell factor [SCF], or kit ligand [KL]), and interleukin-3 (IL-3). Finally, our results show that genetic differences in HGF responses can be preserved after transformation by myb, which may allow dissection of the molecular basis for such differences.

**MATERIALS AND METHODS**

**Growth factors and cytokines.** Recombinant GM-CSF and CSF-1 secreted by engineered yeast were used as crude culture supernatants with activities of $4 \times 10^6$ and $2.5 \times 10^5$ U/mL, respectively, and were kindly provided by Drs N. Gough and T. Wilson (Walter and Eliza Hall Institute, Melbourne, Australia). IL-3 was produced by a baculovirus expression vector and used as crude culture supernatants with activities of $4 \times 10^5$ U/mL, and “Units” of IL-3, GM-CSF, and CSF-1 are defined here such that 50 U/mL gives 50% of maximal colony formation by 50,000 normal murine bone marrow (BM) cells plated in semisolid culture medium. Purified recombinant SLF (MGF) was kindly provided by Dr D. E. Williams (Immunex Corp, Seattle, WA). Recombinant murine tumor necrosis factor-α (TNFa) was a gift from the Ernst-Boehringer-Institut Fur Arzneimittel-Forschung (Vienna, Austria). Recombinant IL-4 purified from engineered Escherichia coli was kindly provided by Dr R. Kastelein (DNAX Research Institute, Palo Alto, CA).

**Transformation of murine fetal liver cells with myb retrovirus.** Murine fetal liver cells were isolated from day 14 fetuses of each of the mouse strains used (CBA; C57B6, referred to subsequently as C57; C3H,HeJ, referred to as C3H; and A/J) and infected with the recombinant myb retrovirus RED(CT3myb) as previously described, with the following variations. Infections were performed by cocultivating $10^6$ irradiated (25 Gy) virus-producing V cells with $10^6$ fetal liver cells. This was performed in the presence of GM-CSF and IL-3 each at 400 U/mL except in experiments where the cells were to be cultured subsequently in factor combinations including SLF; in these cases only IL-3 was added. (The addition of GM-CSF to cocultures is probably irrelevant because this factor is produced at significant levels by the V cells.)

**Culture of MTHCs.** After infection, the fetal liver cells were harvested, washed twice in phosphate-buffered saline (PBS), and resuspended initially in 5 mL of Dulbecco’s modified Eagle medium (DME at containing antibiotics, 20% fetal calf serum (FCS), and HGFs either singly or in combination as indicated in Results for each experiment. HGFs were used at the following concentrations: GM-CSF, 400 U/mL; IL-3, 500 to 800 U/mL; CSF-1, 250 U/mL; SLF, 40 ng/mL. Cells were passaged by dilution into fresh medium to maintain cell densities between approximately $5 \times 10^5$ and $10^6$/mL. In some cases, as indicated, the cells were cultured in GM-CSF in the presence of a feeder layer of irradiated NIH3T3 fibroblasts; these cultures were split and added to fresh 3T3 cells at weekly intervals. These transformed cell populations were not used in subsequent experiments until at least 2 weeks after infection to ensure that there were no normal, ie, untransformed, cells remaining; this was confirmed by monitoring parallel mock-infected cultures in which proliferation ceased and complete terminal differentiation followed.

**Colony formation and characterization.** Colony formation was assessed by plating cells at 5,000/mL in Iscove’s modified Dulbecco medium (IMDM) containing 20% FCS and 0.3% agar and scoring colonies containing greater than 50 cells after 7 days’ incubation. Colony assays contained HGFs at the following concentrations: GM-CSF at 400 U/mL; IL-3 at 400 to 500 U/mL; CSF-1 at 500 to 800 U/mL; SLF at 40 ng/mL (in some experiments different concentrations were used but these always exceeded saturating levels). Morphology of cells within colonies was assessed by drying down the agar cultures, fixing with methanol, staining with May-Grünwald-Giemsa, and microscopic examination.

**3H-thymidine incorporation assay for cell proliferation.** Two thousand cells were incubated for 48 hours in the wells of a 96-well microtiter plate containing 200 μL of culture medium. During the last 6 hours 0.5 μCi of 3H-thymidine was added, after which the cells were harvested and the incorporated radioactivity determined by liquid scintillation counting.

**Surface marker analysis.** Rat monoclonal antibodies (MoAbs) to the surface markers Mac-1, F4/80, CD4, CD8, Gr-1 (8C5), J1 d.2, Pgp-1 (IMT), Thy-1.2, Sca-1, and B220 were used at previously determined optimal concentrations to stain $10^6$ cells in 50 μL for 30 minutes at 4°C. The cells were washed with buffered saline containing 2% FCS and bound antibody was detected by staining as above with fluorescein-conjugated antirat IgG (Silenius Laboratories, Melbourne, Australia) followed by flow cytometry using a Coulter Profile II (Hialeah, FL) or a Becton-Dickinson (San Jose, CA) FACScan. Staining with the fluorescein-conjugated antirat IgG alone was used routinely as a negative control, but on several occasions the validity of this was confirmed by using known negative primary antibodies.

**Induction of differentiation by TNF and IL-4.** Briefly, differentiation of MTHCs was induced by transferring the cells into medium containing TNFα at 500 U/mL and IL-4 at 200 U/mL. Full details and characterization of MTHC differentiation induced by these factors will be presented elsewhere (A.J.H., G. De Cello, P.V.T., and T.J.G., manuscript in preparation).

**RESULTS**

**Generation of MTHC populations.** The experiments described in this report were performed using populations of MTHCs derived by infecting hematopoietic cells from murine fetal liver with the strongly transforming RED(CT3myb) retroviral vector essentially as described previously. In those studies, the cells were cultured in the
presence of GM-CSF. However, as shown below, MTHCs could respond to several different HGFs, and so MTHCs were generated in some cases by culturing the cells in other HGFs or combinations thereof. To examine the responses of MTHCs to IL-3, and more generally, to reduce the influence of possible strain-specific phenomena, we performed experiments with cells from three different mouse strains (C3H, CBA, and A/J) that differ in their sensitivity to IL-3 (see below). Most experiments were performed with uncloned populations to maximize the generality of the results obtained, ie, to reduce the chance that the properties we observed reflect only rare clones. Moreover, isolation and expansion of clones—which is necessarily done at relatively low cell density—may select cells with particular properties because, as we show elsewhere (E.M.M. and T.J.G., submitted), MTHCs exhibit density-dependent growth. Nevertheless, clones were isolated in some cases to check whether the heterogeneity observed in the expression of certain markers was simply caused by clonal heterogeneity.

HGF responses of MTHCs. The clonal MTHC lines described previously could be propagated in the presence of GM-CSF alone and indeed in subsequent experiments, MTHCs could be derived and maintained in the presence of this factor alone. However, we observed that (even in the presence of GM-CSF) proliferation of recently derived transformants could be enhanced by maintaining cells either on feeder layers of irradiated fibroblasts or at relatively high cell densities. This suggested that additional factors may be necessary to support MTHC growth. While the nature and properties of the factors produced under these conditions are explored elsewhere (E.M.M. and T.J.G., submitted), these observations led us to test the effects of a number of HGFs, either alone or in combination, for their ability to support the proliferation of MTHCs. The factors tested were primarily those known to act on myeloid progenitor cells and/or to be produced by fibroblasts.

The various factors were tested in either liquid or semisolid culture media, or both; in no cases were factors found that were active in only one of these systems. Functionally, the factors tested could be grouped into three classes: (1) those which by themselves could stimulate MTHC proliferation; (2) those that could stimulate MTHCs in combination with class (1) factors; and (3) those that could not stimulate MTHC proliferation either alone or in combination with class 1 factors. Class 3 HGFs/cytokines included ILs 1, 4 (but see below), 5, 6, 8, 10, 11; G-CSF; TNF (see also below); erythropoietin; and leukemia inhibitory factor (LIF) (data not shown). The only factors that could act when added alone (class 1) were GM-CSF, IL-3, and, to a lesser extent, CSF-1 (M-CSF). Finally, the class 2 factors identified were CSF-1 and Steel factor (SLF) (also known as stem cell factor [SCF] or mast cell growth factor [MGF]).

Table 1 shows representative data with regard to stimulation of colony formation by the class 1 and 2 factors on MTHCs derived from three different mouse strains (CBA, C3H, and A/J). While in each case the cells used in these experiments were generated and propagated in the presence of GM-CSF, MTHCs could be derived also by culture in the presence of IL-3 plus CSF-1, IL-3 alone (in some cases; see below) and IL-3 plus SLF; in general the pattern of HGF responses obtained with such lines (in a more limited number of experiments) was similar to that seen with MTHCs cultured in GM-CSF. These strains were chosen because of their differential responses to IL-3; cells from C3H and CBA mice respond strongly to IL-3 whereas A/J cells show only a minimal response. Despite a considerable degree of quantitative variation between experiments, particularly in regard to plating efficiency, the data show that GM-CSF alone stimulated colony formation in all cases, and that both CSF-1 and SLF were capable of synergistically enhancing colony formation. We did not detect any synergistic effects of IL-3 and GM-CSF (data not shown). IL-3 alone was a strong stimulus for cells from C3H mice, a less effective stimulus for cells from CBA mice (although in some experiments the responses were greater than that seen in experiment 1 here; data not shown), and failed to stimulate colony formation by A/J cells. Particularly striking was the synergy between IL-3 and CSF-1 observed with all strains. The response to CSF-1 alone was highly variable, and colonies generated were generally much smaller and more diffuse than those obtained with other HGFs or combinations of HGFs (data not shown).

The poor response of A/J cells to IL-3 was also apparent from their response to varying concentrations of IL-3 as measured by 3H-thymidine incorporation (Fig 1), as also reported by Morris et al for normal BM cells. While the responses of MTHCs from each strain to GM-CSF were similar, maximum stimulation by IL-3 was 48% of that obtained with GM-CSF in the case of C3H-derived MTHCs but only 15.7% relative to GM-CSF for A/J MTHCs (note that this may be an overestimate due to the production of CSF-1 by MTHCs [E.M.M. and T.J.G., submitted], which can synergise with IL-3). However, the 50% of the maximal response to IL-3 was obtained at similar IL-3 concentrations in each case, suggesting that in agreement with previous work, the “defect” in A/J is not caused by lower IL-3 receptor numbers or affinity. Despite this poor response to IL-3 alone, indicated also by the failure to derive cell lines from this strain, synergy between IL-3 and CSF-1 was detectable with MTHCs from A/J fetal liver, although to a lesser degree than seen with C3H and CBA cells (Table 1); this again is consistent with the response of A/J marrow cells to this combination of HGFs. In fact, cell lines could be derived from A/J fetal liver by culturing the virus-infected cells in the presence of IL-3 plus CSF-1 but not IL-3 alone, whereas C3H cell lines could be derived in both cases. In general, the synergy detected between GM-CSF or IL-3 and CSF-1 was greater than that seen with the former two factors and SLF. Furthermore, three-factor combinations of GM-CSF or IL-3 plus both CSF-1 and SLF showed further synergistic effects (data not shown).

Phenotype of MTHCs. Our previous characterization of clonal, GM-CSF-dependent MTHC lines derived from murine fetal liver indicated that they belonged to the GM sublineage on the basis of morphology and expression of the cell surface antigens Mac-1 and Grm3.2. Table 2 shows a more comprehensive analysis of a range of surface markers expressed on MTHCs using cells from three mouse strains,
and Fig 2 shows several representative analyses of expression of these and other markers. The data summarize a large number of experiments in which transformed cells were generated by infecting fetal liver cells with the RED(CT3myb) retrovirus and subsequent culture in either GM-CSF or in other factors or combinations that were shown above to be active in stimulating MTHC proliferation. While there is a degree of variability in the expression of some markers, the expression pattern confirms that the MTHCs resemble early GM progenitors. Thus, almost all the populations examined express both the Mac-1 and CD4 markers, which are both exclusive to the GM lineage, as well as the F4/80 macrophage marker and, in the case of cells from C57 mice (Fig 2C), the 7/4 granulocyte marker. In contrast, neither the B-cell marker B220 nor either of the T-cell markers CD8 or CD3 were detected on these cells (data not shown). When low levels of CD4 expression were detected on some populations (eg, see Fig 2A), this may reflect expression of this classical T-cell marker on murine macrophages, because CD4 expression is well documented on human and rat macrophages; while expression was not detected on murine macrophages in a parallel study, we have nevertheless detected low levels on such cells (A.J.H., unpublished observations, August 1990).

Alternatively, low levels of CD4 expression could reflect an immature phenotype because it has recently been reported that this marker is expressed at low levels by repopulating cells as well as spleen colony-forming cells (CFU-S) and most myeloid progenitors. Expression of Pgp-1/CD44 on all cell lines tested was not as surprising as it has been detected on a wide range of hematopoietic cells ranging from granulocytes to CFU-S, but not on most thymocytes. While roughly half of the cell lines tested are shown as positive in Table 1, expression in most of these cases was rather weak (data not shown). Even so, the pattern of J 1 Id expression is rather hard to interpret. This marker is expressed on more than 90% of BM and fetal liver cells, but not on mature T cells or monocyte/macrophages nor on CFU-S or repopulating cells. Moreover, it is expressed on at least one myeloid cell line (M1) but not on three others (FD C-P1, WEHI-3B, or 32D). That MTHCs resemble early progenitor cells is more clearly indicated by the observation that they generally express the Thy-1 marker (Table 1 and Fig 2), which has been detected on populations highly enriched for hematopoietic stem cells and on early progenitor cells. Furthermore, when target cells from an appropriate mouse strain (C57) were used, a substantial fraction of the cells were positive for the Sca-1 antigen (Fig 2C), which has also been used to isolate early stem/progenitor cells.

Expression of both the Thy-1 and Sca-1 markers was heterogeneous in most of the populations examined (Fig 2) and in both cases expression was lost when cultures were transferred to NIH3T3 feeder layers (Table 2 and Fig 2B). Sca-1 expression was also influenced by exogenous HGFs because highest levels were seen on cells cultured in the presence of IL-3, while culture in GM-CSF alone resulted in low but detectable levels of Sca-1 (data not shown). The heterogeneity of Thy-1 expression (see Fig 2 for examples), was not simply caused by clonal variation in the populations examined because heterogeneity was observed even in the case of clonal cell lines (Fig 2D; note that although the clones depicted were derived by infection with RED(CT2myb) rather than RED(CT3myb), we have not observed any consistent or significant differences in the surface phenotypes of cells transformed by these two myb constructs). However, alterations in Thy-1 expression appear to be reversible in some cases because transfer of cells from feeder layers to GM-CSF alone resulted in restoration of Thy-1 expression (data not shown). Moreover, cell sorting experiments showed that both Thy-1-positive and Thy-1-negative subpopulations from heterogeneously expressing lines could regenerate both phenotypes on subsequent culture (unpublished observations, October 1991). The significance of and mechanisms involved in this phenomenon are being investigated further.

### Table 1. Colony Formation by MTHCs in Various Combinations of HGFs

<table>
<thead>
<tr>
<th>Expt†</th>
<th>Strain</th>
<th>Plated′</th>
<th>GM + CSF-1</th>
<th>GM + SLF</th>
<th>IL-3 + CSF-1</th>
<th>IL-3 + SLF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CBA</td>
<td>47</td>
<td>5</td>
<td>50</td>
<td>70</td>
<td>29</td>
</tr>
<tr>
<td>2</td>
<td>CBA</td>
<td>3.5</td>
<td>0.5</td>
<td>24</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CBA</td>
<td>387</td>
<td></td>
<td>599</td>
<td>564</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C3H</td>
<td>183</td>
<td>83</td>
<td>278</td>
<td>800</td>
<td>&gt;218</td>
</tr>
<tr>
<td>5</td>
<td>A/J</td>
<td>80</td>
<td>0.7</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>C3H</td>
<td>120</td>
<td>55</td>
<td>161</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>C3H</td>
<td>42</td>
<td>0</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>A/J</td>
<td>157</td>
<td>33</td>
<td>689</td>
<td>&gt;1,000</td>
<td>618</td>
</tr>
<tr>
<td>7</td>
<td>A/J</td>
<td>28</td>
<td>42.5</td>
<td>50</td>
<td>188</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells were plated at 5,000/mL, except in Expt 5, where they were plated at 12,500/mL.
† Where the same experiment number is indicated, transformation, culture, and plating were all performed in parallel.

**Differentiation potential of MTHCs.** Because the surface phenotype and HGF responses of MTHCs were consistent with those of a myeloid progenitor cell, we were interested to determine the differentiation potential of the MTHCs. Inspection of stained cytospin preparations showed the presence, in addition to the typical immature myeloid or "myeloblast-like" cells, of more differentiated cells of both the granulocytic and monocytic lineages. While the extent of this spontaneous differentiation was rather...
PROPERTIES OF myb-TRANSFORMED MYELOID CELLS

variable, examination of several independently derived MTHC populations indicated that monocyte/macrophages were present at frequencies between 0 and 5.9% (average, 2.8%), whereas granulocytes (including band and segmented forms) comprised 0 to 8% (average, 1.8%). In addition, the presence of elongate, adherent cells in some cultures (particularly slightly overgrown cultures) also suggested that MTHCs could differentiate to macrophages, as also suggested by the presence of a small fraction of phagocytic cells in several MTHC lines described previously.19 Examination of colonies formed in semisolid medium was also consistent with a degree of spontaneous granulocytic and monocytic differentiation of MTHCs. Table 3 shows that the majority of colonies formed in the presence of GM-CSF or IL-3 plus CSF-1 contained macrophages as identified both by morphology and esterase staining (data not shown), whereas some colonies also contained granulocytes. Variable numbers of cells in each colony retained an immature phenotype.

A number of HGFs and cytokines were tested for their ability to promote morphologic differentiation; these included G-CSF, CSF-1, erythropoietin, IL-5, γ-interferon, and transforming growth factor-β. None of these induced substantial differentiation to macrophages, granulocytes, or...
other cell types. We also tested chemical agents known to induce differentiation of other myeloid cell lines; again these—the phorbol ester PMA, dimethyl sulphoxide, retinoic acid, and dihydroxy-vitamin D—were also ineffective and/or toxic. The only effective agent found was TNFα, which has previously been shown to induce monocytic differentiation of the myeloid leukemia cell lines HL-60 and the JCS subline of WEHI-3B. IL-4 synergized strongly with TNFα in inducing differentiation of MTHCs, as is also the case for WEHI-3B/JCS (K.-N. Leung, N.K. Mak, M.C. Fung, and A.J.H.: Immunology [in press]), but was ineffective alone. We have found recently that TNFα plus IL-4 can also induce differentiation to dendritic cells if low levels of GM-CSF are maintained; a complete analysis of the induction of MTHC differentiation by TNF and IL-4 will be described in detail elsewhere (A.J.H., G. De Cello, P.V.T., and T.J.G., manuscript in preparation). Figure 3 shows the appearance under the phase-contrast microscope of MTHCs grown in the presence of GM-CSF (panel A) or treated with TNFα plus IL-4 in the absence of GM-CSF (panel B). It can be seen that while the undifferentiated cells in GM-CSF are round and highly refractile, the TNFα plus IL-4-treated cultures contain a high proportion of cells that adhere to and flatten out on the culture surface, and are much less refractile and more elongate in appearance. That these macrophage-like cells do in fact reflect true macrophage differentiation has been confirmed by esterase staining, phagocytosis assays, and surface marker analysis (A.J.H., G. De Cello, P.V.T., and T.J.G., manuscript in preparation). Because the cultures depicted in Fig 3 were initiated with equal numbers of cells, it is also apparent that TNFα/IL-4 treatment results in a marked inhibition of proliferation as might be expected to accompany terminal differentiation. Inhibition of proliferation was also indicated by the complete absence of mitotic figures in the treated populations, compared with a mitotic index of 4% in samples grown in GM-CSF alone. Thus, MTHCs can be switched from a rapidly proliferating, immature phenotype to a differentiated phenotype with reduced proliferative capacity by altering their HGF/cytokine environment. Finally, it is worth noting that while the majority of cells in liquid culture retained an immature phenotype (as described previously), most of the colonies formed in semi-solid medium contained a substantial proportion of more differentiated cells, i.e., macrophages (Table 3). This suggests that the MTHCs may secrete an autocrine factor capable of enhancing differentiation, and that effective concentrations may only be reached when cells are in very close proximity, as in colonies. However, it is unlikely that differentiation in colonies is caused by autocrine TNFα production because preliminary polymerase chain reaction (PCR) analyses have not detected TNFα mRNA (unpublished observations, January 1993).

### DISCUSSION

Our characterization of murine hematopoietic cells transformed by activated c-myb has shown that in many respects they resemble early GM progenitors. The potential of MTHCs to also generate dendritic cells is consistent with this, because a recent report has shown that granulocytes, macrophages, and dendritic cells share a common progenitor. However, they differ from their normal counterparts in that they retain these characteristics and remain in a continuously proliferating state, provided that appropriate HGFs are present. Nevertheless, they appear to undergo a degree of spontaneous granulocytic and monocytic differentiation, with the latter being inducible almost to completion by TNFα plus IL-4. This ability to switch MTHCs from a proliferative state to one of monocytic/dendritic differentiation (A.J.H., G. De Cello, P.V.T., and T.J.G., manuscript in preparation) should provide a useful system for studying the molecular events that mediate these two processes. Moreover, because MTHCs have undergone (as far as we know) only one genetic event that alters their behavior, i.e., the constitutive expression of myb activity, many of these processes should be essentially identical to those in normal cells. Indeed, because myb encodes a transcription factor, it is likely to act downstream of HGF receptors and their intracellular signaling pathways. In contrast, most other myeloid cell lines that exhibit inducible differentiation have completely

### Table 2. Surface Marker Analysis of MTHCs

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Strain</th>
<th>BC5</th>
<th>Mac-1</th>
<th>F4/80</th>
<th>J11d</th>
<th>Thy-1</th>
<th>Pgp-1</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM + CSF-1 CBA</td>
<td>+/[1/1]</td>
<td>+/[1/1]</td>
<td>ND</td>
<td>ND</td>
<td>−/[1/1]</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>GM + 3T3+ CBA</td>
<td>+/[1/1]</td>
<td>+/[2/2]</td>
<td>ND</td>
<td>ND</td>
<td>−/[2/2]</td>
<td>ND</td>
<td>−/[1/1]</td>
<td></td>
</tr>
<tr>
<td>IL-3 C3H</td>
<td>+/[1/2] 1</td>
<td>+/[1/1]</td>
<td>+/[1/2]</td>
<td>+/[1/2]</td>
<td>+/[1/2]</td>
<td>+/[2/2]</td>
<td>+/[1/2]</td>
<td></td>
</tr>
<tr>
<td>IL-3 + CSF-1 A/J</td>
<td>ND</td>
<td>ND</td>
<td>+/[1/1]</td>
<td>−/[1/1]</td>
<td>−/[1/1]</td>
<td>−/[1/1]</td>
<td>−/[1/1]</td>
<td></td>
</tr>
<tr>
<td>IL-3 + SLF C3H</td>
<td>+/[1/1]</td>
<td>+/[1/1]</td>
<td>ND</td>
<td>ND</td>
<td>+/[1/1]</td>
<td>ND</td>
<td>−/[1/1]</td>
<td></td>
</tr>
</tbody>
</table>

All HGFs were used at saturating concentrations (see Materials and Methods). "GM-CSF" is abbreviated as "GM" in this table.

1 Populations showing levels of fluorescence greater than that obtained with control antibodies were scored as positive.

1 Cells were cultured on a feeder layer of irradiated NIH3T3 cells.
lost their dependence on HGFs for survival and proliferation, and frequently require nonphysiologic agents to induce differentiation.

MTHCs respond to HGFs and combinations of HGFs that act on normal myeloid progenitors. Synergistic responses to combinations of HGFs are a characteristic of the earliest progenitor cells, which are termed high-proliferative potential colony-forming cells (HPP-CFC). HPP-CFCs have been divided into several classes on the basis of their appearance after 5-fluorouracil treatment of BM, which pre-
Table 3. Presence of Differentiated Cells in MTHC Colonies

<table>
<thead>
<tr>
<th>Expt: Strain</th>
<th>Factors</th>
<th>% Colonies Containing*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>1: A/J</td>
<td>GM-CSF</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>IL-3 + CSF-1</td>
<td>70</td>
</tr>
<tr>
<td>2: C3H</td>
<td>GM-CSF</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>IL-3</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>CSF-1</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>IL-3 + CSF-1</td>
<td>88</td>
</tr>
</tbody>
</table>

Abbreviations: M, predominantly macrophage; G, predominantly granulocyte; G&M, granulocyte plus macrophage; Mixed, contains a substantial fraction of undifferentiated cells plus granulocytes and macrophages; None, no recognizable differentiated cells.

* The figures are the percentages of colonies in which cells of the indicated morphologic phenotype could be detected.

Table 3. Presence of Differentiated Cells in MTHC Colonies

<table>
<thead>
<tr>
<th>Expt: Strain</th>
<th>Factors</th>
<th>% Colonies Containing*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>1: A/J</td>
<td>GM-CSF</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>IL-3 + CSF-1</td>
<td>70</td>
</tr>
<tr>
<td>2: C3H</td>
<td>GM-CSF</td>
<td>83</td>
</tr>
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<td></td>
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</tbody>
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sumably reflects their hierarchical proximity to the most primitive stem cells. These classes also show requirements for different numbers and combinations of HGFs, with earlier classes needing increasing numbers of different HGFs. HPP-CFCs that respond to the same combinations of HGFs as MTHCs have been described and termed HPP-CFC.

The expression pattern of the various surface markers also suggests a similarity between MTHCs and normal early progenitors. Thy-1 and Sca-1 (and CD4) are both expressed on stem cells as well as early progenitor cells (including at least some HPP-CFC), but the expression of granulocytic and monocytic differentiation markers such as 8C5, 7/4, F4/80, and Mac-1 are characteristic of the GM lineage but not generally thought to be on multipotential or stem cells. Indeed, removal of cells expressing these lineage markers is frequently used to purify primitive hematopoietic cells. Consequently, one might assume that this combination of markers conclusively places MTHCs in an early committed GM progenitor compartment. However, it is not clear whether progenitors with this combination of markers actually exist in normal BM or fetal liver. Such cells may exist because one report describes primitive progenitors that express a combination of Thy-1, B220, and Mac-1, and regenerating BM (ie, after 5-fluorouracil treatment) HPP-CFC express several of these “lineage” markers (I. Bertoncello, personal communication, January 1993). On the other hand, it must be remembered that transformation by myb clearly affects the differentiation of the infected fetal liver cells, so it is quite conceivable that they display an aberrant combination of markers. Such phenomena have been reported on many leukemic cells. More specifically, chicken myeloid cells transformed by v-mybAMV have been characterized as displaying a combination of immature and more mature markers not found on normal cells; this phenotype appears to be “enforced” by myb itself. In addition, chicken cells transformed by E26, which encodes an myb-ets fusion protein, exhibit characteristics of erythroid progenitors but have recently been shown to be multipotential in that they can be induced to undergo both myeloid and eosinophilic differentiation. Thus, it is possible that the MTHC phenotype also represents an abnormal combination of late and early markers. If this is the case, it is also possible that MTHCs may retain some potential to give rise to progeny other than cells of the GM lineage. In fact, preliminary studies have shown the presence of a very small fraction (≤1%) of cells that exhibit erythroid and megakaryocyte morphologies and that are associated with adherent macrophages generated by spontaneous differentiation of MTHCs. We have not yet confirmed the identity of these cells with specific stains and other markers, nor have we been able to increase this fraction by treatment with HGFs that might be expected to favor differentiation along these.

Fig 3. Induction of monocytic differentiation of MTHCs by TNFα plus IL-4. MTHCs were cultured for 3 days in GM-CSF (250 U/mL) (A) or TNFα (500 Units/mL) plus IL-4 (200 U/mL) (B), and photographed under a phase-contrast microscope.
lineages (e.g., erythropoietin). However, if these preliminary observations can be verified with clonal cell lines, they will provide strong evidence that MTHCs are in principle multipotential.

Although terminal differentiation of MTHCs does not occur in the presence of HGFs, the presence of particular HGFs can still influence the expression of certain markers. Expression of the early markers Thy-1 and Sca-1 is lost when MTHCs are cultured on fibroblast feeder layers (Fig 2, B and C), and is decreased on transfer from IL-3 plus SLF to GM-CSF (Fig 2A and data not shown). Similarly, reciprocal changes in the macrophage marker Mac-1 expression have also been observed (e.g., Fig 2A). Such changes are suggestive of the MTHCs assuming a somewhat more mature phenotype; it is interesting that these occur when cells are transferred to an environment of HGFs that act principally on more mature cells than those in which the cells were originally cultured. Thus, the combination of IL-3 plus SLF is believed to act on progenitors that precede those influenced by GM-CSF, and CSF-1, which we believe is primarily responsible for at least some of the effects of fibroblasts on MTHCs (E.M.M. and T.J.G., submitted), acts on more committed macrophage progenitors. Some caution is necessary in the precise interpretation of these data, though, because the use of uncloned cell lines formally allows the possibility that the changing HGF environments may favor outgrowth of subpopulations representing different differentiation states, rather than directly altering marker expression.

In conclusion, our characterization of MTHCs has shown that they exhibit properties that should enable them to be used as a model for studying several aspects of hematopoietic regulation. While they behave as continuously proliferating myeloid progenitors when cultured in the presence of appropriate HGFs (e.g., GM-CSF), they can be induced to undergo monocyteic differentiation by treatment with TNFs plus IL-4, suggesting that they will be useful for studying signaling pathways and other molecular events involved in the "switch" between proliferation and differentiation. In addition, the fact that MTHCs retain the genetically determined sensitivities to IL-3 of the mouse strains from which they are derived indicates that they may prove valuable in dissecting the biochemical/molecular basis of this variation and hence provide insight into the mechanism of IL-3 signaling. Another aspect of HGF signaling for which MTHCs may be useful is in studying the mechanistic basis of HGF synergy, as we have shown here that they respond to combinations of HGFs known to act on certain normal progenitors. The cell lines from A/J mice should be particularly useful here too because they retain the ability to respond to the combination of IL-3 and CSF-1 as do BM cells from this strain.33 In this regard also, their responses to HGFs known to exist in both membrane-bound and soluble forms—i.e., SLF and CSF-1—and to be produced by hematopoietic-supporting stromal cells52 has raised the possibility that MTHCs may be of value in modeling some features of interactions between hematopoietic and stromal cells. This possibility has been borne out by other, recent work with these cells (E.M.M. and T.J.G., submitted).

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