Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture

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Summary

When embryonic stem cells are cultured directly in semi-solid media (methyl cellulose), they proliferate and differentiate to generate colonies known as embryoid bodies (EBs). These EBs consist of differentiated cells from a number of lineages including those of the hematopoietic system. Following 10 days of culture in the presence of 10 % fetal calf serum, more than 40 % of all EBs from three different ES cell lines, CCEG2, D3 and SQ1.2S8 contained visible erythropoietic cells (i.e. red with hemoglobin). βH1 (z globin) mRNA is detectable in EBs within 5 days of differentiation, whilst β^{maj}-globin RNA appears by day 6. In the presence of erythropoietin (Epo), the frequency of EBs with erythropoietic activity increases to greater than 60 %; Epo also prolongs this erythropoietic activity. Interleukin-3 (IL-3) does not significantly increase the frequency of EBs that contain erythroid cells, but increases slightly the number of erythropoietic cells associated with them. In the presence of IL-3, in addition to cells of the erythroid lineage, macrophages, mast cells and in some instances neutrophils are found within differentiating EBs. The development of macrophages is significantly enhanced by the addition of IL-3 alone or in combination with IL-1 and M-CSF or GM-CSF. When well-differentiated EBs are allowed to attach onto tissue-culture plates and grown in the presence of IL-3, a long-term output of cells from the mast cell lineage is observed. These findings indicate that early hematopoietic precursor cells can be readily generated from ES cells under well-defined culture conditions, and thus provides a unique in vitro model with which to analyze the earliest events involved in the development of the hematopoietic system.

Key words: Embryonic stem cells, hematopoietic system, erythropoitin, interleukin-3.

Introduction

Mouse embryonic stem (ES) cells are continuously growing cell lines derived from the inner cell mass of 3.5 day blastocysts (Evans and Kaufman, 1981; Martin, 1981). A unique feature of these cells is that they can be maintained in vitro as totipotent cell lines for many generations. When reintroduced back into the mouse blastocyst, ES cells will contribute to all lineages of the mouse (Bradley et al. 1984). In addition, when allowed to form three-dimensional structures known as embryoid bodies (EBs), ES cells will differentiate spontaneously into many cell types including those of the hematopoietic system (Evans and Kaufman, 1981; Martin, 1981; Doetschman et al. 1985). The ability to generate differentiated progeny from a continuously growing stem cell population in vitro provides a unique system with which to analyze both the cellular and molecular events involved in the first stages of lineage determination. The hematopoietic system, in particular, is ideal for these studies since many lineages are already well characterized, precursor cells representing various stages of development within these lineages can

be detected by in vitro clonal assays and a large number of recombinant growth factors that act at different stages in the system are available (Metcalf, 1977; Metcalf, 1984; Nicola, 1989). To date however, the only hematopoietic cells identified within these differentiated EBs have been erythroid and these at a low frequency. Furthermore, the appearance of these hematopoietic cells has been dependent upon uncharacterized and highly variable components, for example human cord serum (Doetschman et al. 1985). The aim of this work is to examine the various elements involved in the generation and expansion of (embryonic) hematopoietic cells in vitro from ES cells.

To define better the factors and conditions that influence the early stages of hematopoiesis, we used a semi-solid culture system that provides a three-dimensional matrix to support the developing EB and which efficiently and reproducibly promotes the differentiation of ES cells to hematopoietic cells. In this report, we describe this system and demonstrate that more than 50 % of all EBs formed under these conditions produce hematopoietic cells. Significant amounts of both embryonic z globin (βH1) and adult β

major globin RNAs are detectable early in this differentiation. Moreover, we show that, in addition to erythroid cells, differentiating ES cells are capable of giving rise to macrophages, neutrophils and mast cells. Finally, using this system, we have analyzed the effects of a number of hematopoietic growth factors on the early stages of hematopoietic development.

Materials and methods

Cells

A G418 resistant subclone (CCEG2) of the 129/Sv-derived ES line CCE (Robertson et al. 1986), adapted to grow in the presence of LIF and without feeder cells, was used in this study. To maintain the cells in an undifferentiated state, they were subclutured regularly onto gelatin (0.1% swine skin ~300 bloom)-treated tissue culture flasks in Dulbecco's modified Eagles media (DME) supplemented with 15% fetal calf serum (FCS; selected batch), $1000\,units\,ml^{-1}$ LIF (supplied by Amrad Co Ltd., Victoria, Australia) and $1.5\times10^{-4}\,M$ $(12.6\,\mu l\,l^{-1})$ monothioglycerol (MTG). Under these conditions, more than 95% of the cell population remains undifferentiated as determined by visual inspection under phase-contrast microscopy. One to five passages before the initiation of differentiation experiments, cells were transferred into Iscoves modified Dulbecco's media (IMDM; Gibco-BRL). The ES cell line D3 was originally derived from a 129/Sv mouse (Doetschman et al. 1985); the subclone used for this study is a LIF-dependent, feeder-free variant (Williams et al. 1988) and was maintained under conditions identical to those described for CCEG2. The ES cell line SQ1.2S8 is a subclone of SQ1.2, which was established from a male C57Bl/6 blastocyst (M.V.W. unpublished) using conventional techniques (Robertson, 1987). SQ1.2S8 is cultured on irradiated (2000 rad) STO fibroblasts (Ware and Axelrad, 1972) feeders in the same media as described for CCEG2.

Differentiation conditions

For the differentiation of CCEG2 and D3 ES cells, rapidly growing, healthy cells were dissociated by trypsinization (0.25% trypsin in phosphate-buffered saline with 1mM EDTA) and cultured in 0.9% methyl cellulose (methyl cellulose MC USP 4000 mPa.s., Fluka A.G., Buchs, Switzerland) in IMDM supplemented with 10% FCS. Cells were cultured in a final volume of 1.25 ml in 35 mm diameter bacterial grade Petri dishes (Greiner). The final cell concentration used is dependent to some extent on the ES cell line and the length of time it has been in culture. CCEG2 were routinely plated at 0.9 to $1.5\times10^3\,cells\,ml^{-1}$. D3 were plated at 1 to $2\times10^3\,cells\,ml^{-1}$. All cultures are supplemented with additional MTG at initiation to give a final concentration of $4.5\times10^{-4}\,M$ $(37.8\,\mu l\,l^{-1})$ and, where indicated, $50\,\mu g\,ml^{-1}$ ascorbic acid (dissolved in water at $50\,mg\,ml^{-1}$) or $50\,\mu g\,ml^{-1}$ vitamin E (dissolved in ethanol at $50\,mg\,ml^{-1}$) are also included.

The ES cell line SQ1.2S8 has a low plating efficiency when put directly into methyl cellulose media. To increase the plating efficiency SQ1.2S8 cells are grown initially (48 h) in suspension at $4\times10^5\,cells\,ml^{-1}$ in IMDM supplemented with 10% FCS and $4.5\times10^{-4}\,M$ MTG. The aggregates that form are then added to methyl cellulose media where they develop into EBs.

Expansion and identification of mast cells and macrophages in 'liquid cultures'

Three to ten EBs grown in methyl cellulose cultures for 15 days are transferred onto 35 mm diameter tissue culture plates with IMDM, 10% FCS and either IL-3 alone (for mast cells) or a combination of IL-3 and M-CSF (for macrophages). Rapidly growing mast cells develop within 7–14 days in liquid culture and can be maintained for at least 8 weeks by passaging and/or feeding as required. The cells were identified as mast cells by characteristic staining with May–Grünwald–Giemsa and toluidine blue. In most instances, macrophages developed within the EBs in methyl cellulose cultures. These cells could be maintained and expanded in the liquid cultures in the presence of IL-3 and M-CSF.

Macrophages were identified by morphology; cells were cytospun, air-dried, fixed in acetone and stained with May–Grünwald–Giemsa. Cells were also stained with the macrophage-specific antibody F4/80 (Austyn and Gordon, 1981) followed by the $F(ab')_2$ fragment of mouse anti-rat IgG (H+L specific) conjugated to peroxidase (Jackson Immunoresearch Lab.). Peroxidase activity was developed in 0.1% diaminobezidine hydrochloride plus 0.01% H_2O_2. Endogenous peroxidase activity was determined by incubation with the second antibody alone.

Factors

Recombinant human erythropoietin, purchased from Cilag A.G. (Schaffhausen, Switzerland) was used at $2\,units\,ml^{-1}$. Recombinant IL-3 was provided as conditioned medium of the producer X63 Ag8–653 myeloma cells transfected with a vector expressing IL-3 (Karasuyama and Melchers, 1988). A second source of IL-3 was purified mouse recombinant IL-3 kindly provided by the Genetics Institute Inc. (Cambridge, Mass., USA). Recombinant murine M-CSF and GM-CSF were also gifts from the Genetics Institute Inc. L-cell conditioned media was used in some experiments as a source of M-CSF. All factors were used at concentrations which gave at least three times half-maximal colony response in murine bone marrow colony assays. Recombinant human IL-1 alpha was provided by Dr Peter Lomedico (Hoffmann-La Roche, Nutley) and used at $500\,units\,ml^{-1}$. Where factors were present, they were always added at the initiation of the culture.

Scoring of globinisation

EB scoring used a Leitz Diavert microscope fitted with a halogen (white) light source and a pale blue filter, using 12.5×4 objectives and dark-field illumination. All EBs of ~50 μm or greater were counted. Those containing any trace of red colour (hemoglobin) were scored as positive. For each condition, fifty EBs, in duplicate were counted, the number of positives being expressed as a percentage. Comparison with dot blots of RNA made from single EBs, probed with a cDNA of the embryonic globin gene $\beta H1$ (pBSBH1), indicated that this method of scoring was equivalent to, or an underestimation of, the number of EBs expressing $\beta H1$ and/or β^{maj}-globin RNA (data not shown).

Probes

A $\beta H1$ probe (pBSBH1) was derived from a PCR of cDNA synthesized from 13-day mouse yolk sac RNA, using primers matching 339–359 bp and 541–561 bp of the mRNA. The fragment was ligated into the SmaI site of Bluescript (Stratagene). The β^{maj}-globin probe (pBM700) is a HindIII-

NcoI 700 bp fragment, generated from a *SacI-EcoRI* 7 kb genomic clone, GSE428, provided by Dr Frank Grosveld, and was subcloned into Bluescript.

RNAase protection assay

Total cellular RNA was made using the AGPC method of Chomczynski and Sacchi (1987). RNA was quantified by measuring the OD_{260} and by dot–blot hybridization to a mouse cDNA β-actin probe. RNAase protection was performed as described by Sambrook *et al.* (1989). Briefly, βH1 (*Xba*I cleaved pBSBH1; protected fragment 222 bases) or β-major globin (*Bam*HI cleaved pBM700; protected fragments 142 and 97 bases) and a β-actin control (protected fragment 47 bases) radiolabeled RNA probes were generated using the T3 promoter in Bluescript. RNA probes (2.5×10^5 cts min^{-1} of each) were hybridized together with 2 μg of total RNA for 12–16 h at 47°C. RNAase digestion was at 30°C for 60 min with 500 units ml^{-1} RNAase T1 and 50 μg ml^{-1} RNAase A. Protected fragments were analyzed on a 6 % polyacrylamide/7M urea gel and visualized by autoradiography (Sambrook *et al.* 1989). The intensity of signal was quantified directly from the polyacrylamide gel using a Phosphor Imager (Molecular Dynamics) and expressed as a ratio of β-actin expression.

Results

Parameters for the differentiation of ES cells in methyl cellulose

When ES cells are seeded directly into methyl cellulose media they proliferate and generate colonies of differentiated cells (EBs), including those of the hematopoietic system. Under standard conditions (0.9 % methyl cellulose, 10 % FCS and 1.5×10^{-4} M MTG) the early development of EBs was independent of any added growth factors but highly dependent upon the initial cell density. The addition of various hematopoietic growth factors does not significantly influence the plating efficiency. However, increasing the amount of the reducing agent MTG to a final concentration of 4.5×10^{-4} M, significantly increases the plating efficiency (fivefold) and hematopoietic activity, whilst reducing the dependency on initial cell density for growth. Moreover, in the presence of 4.5×10^{-4} M MTG, the kinetics of hematopoiesis becomes extremely consistent between experiments. Other reducing agents, such as ascorbic acid and vitamin E have similar effects; however, the most consistent results are obtained with MTG. It was found however, that the plating efficiency and the kinetics of differentiation have to be established for different ES lines.

When seeded at 1.1×10^3 cells in 1.25 ml methyl cellulose media, CCEG2 consistently generate 50 to 100 EBs. Three days after plating of ES cells directly into methyl cellulose media small colonies of developing ES/EB are seen. Over the next few days the EBs continue to grow, reaching a diameter of 50 μm to greater than 700 μm. As detailed below, the appearance of erythroid cells within the EBs is observed between days 7 and 8 of culture. The plating efficiency of D3 is somewhat lower than that of CCEG2, routinely 1.3×10^3 cells plated under the same conditions gives

rise to 20 to 50 EBs. The feeder-dependent line SQ1.2S8 develops very poorly when seeded directly into methyl cellulose cultures. However, if the initial stages of differentiation are carried out in suspension culture, aggregates form, which when seeded into methyl cellulose develop similarly to CCEG2 and D3.

Having established the parameters that reproducibly promote the growth and differentiation of ES cells in culture, the next series of experiments were aimed at determining the role of various hematopoietic growth factors on the development of hematopoietic cells within the EBs.

Effect of growth factors on the development of hematopoietic cells from ES cells

*Differentiation in the absence of factor*

In the absence of added factors, the first evidence of hematopoiesis is the appearance of nucleated erythroid cells (identified by the red colour of hemoglobin) in EBs between days 7 and 8 of culture. This early phase of erythropoiesis appears as a relatively small red area or glow within the center of the EB. Approximately 3 to 10 % of all EBs contain visible erythroid cells by this time. Over the next three days, globinisation increases swiftly, with a peak of 40 to 60 % of all EBs becoming red. (Fig. 1A). At this later stage, some EBs rupture releasing pale red erythroid cells which remain at the periphery. In general however, the early phase of erythroid cells appears to originate from and remain within the center of the EB in the absence of additional factors (Fig. 2A). This early stage of erythropoiesis is transitory, decreasing rapidly after day 10 of culture.

After 12 to 18 days, 5 to 10 % of EBs develop clusters of five to ten macrophage-like cells (Fig. 1B). The addition of the reducing agents ascorbic acid or vitamin E to the media increases the percent of macrophage scoring colonies to 10–15 %, together with the degree of globinisation. Other hematopoietic lineages are not observed in the absence of added factors.

A similar patten of development is observed in the absence of factors with D3 ES cells seeded directly into methyl cellulose and with SQ1.2S8 ES cells, following a 48 h liquid culture period (see Materials and methods).

Effect of Epo

In the presence of Epo (2 units ml^{-1}), 60 to 70 % of all CCEG2-derived EBs contained erythroid cells by day 12 of culture. The addition of Epo does not influence the kinetics of the appearance of erythropoiesis; however, it markedly increases the number of erythroid cells associated with each EB and prolongs their survival in culture (Fig. 1A). In some instances, the growth of these nucleated erythroid cells is so substantial that a red halo of cells forms around the central EB (Fig. 2B). In the presence of lower concentrations (<1 unit ml^{-1}) of Epo, a reduced level of erythropoiesis occurs. At higher concentrations (>2 to 100 units ml^{-1}), little enhancement is noted with the exception of a possible increase in the intensity of colour at 100 units ml^{-1}. No effect is noted with other myeloid
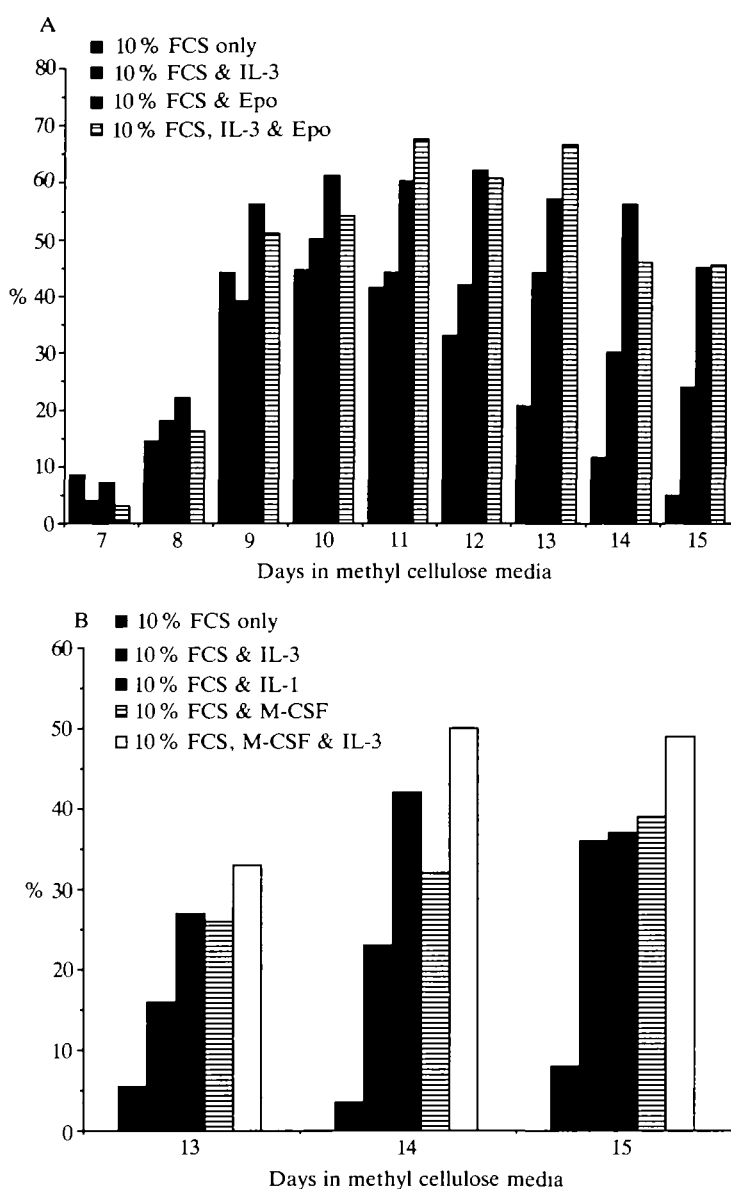
Fig. 1. (A) % EBs 'red' over time; (B) % EBs with macrophage-like cells. For each time point and condition, 50 EBs were counted in duplicate. A shows the percentage of EBs that presented any trace of redness (hemoglobin) with time, ±factors. B shows the percentage of EBs that developed macrophage-like cells at their periphery. A positive was regarded as any EB associated with five or more macrophage-like cells.

lineages over this range. Similar effects for Epo are observed on both D3 and SQ1.2S8.

Effect of IL-3

At the concentrations used, IL-3 alone does not significantly increase the frequency of EBs that produce erythropoietic cells compared to those grown in its absence. The presence of IL-3 does, however, lead to an increase in the number of red cells associated with EBs, as well as prolonging the survival of these cells (Fig. 1A). This effect is not as dramatic as that observed in culture supplemented with Epo. In the presence of

both Epo and IL-3, there is no marked synergism of either erythropoiesis or of the other myeloid lineages examined.

In addition to its effects on the development of the erythroid lineage, IL-3 also influences the development of macrophages from EBs. Approximately 2 to 3 days after the appearance of the erythroid lineage, cells with the morphology of macrophages are seen. Although clusters of 5 to 10 of these cells develop from EBs in the absence of factors, the frequency of EBs that contain these cells and the size of this population is greatly increased, reaching 40 % at 15 days by the addition of IL-3 (Fig. 1B). It was also noted that there is an overlap in the EB population that exhibits extensive erythropoiesis and those that later develop macrophages. There is also an enhancement in the kinetics of the appearance of this population: macrophage-like cells being readily detected between days 9 and 10 of culture (2 days earlier than in the absence of factors).

To determine if these cells are indeed of the macrophage lineage, a number of EBs were pooled and cells stained with F4/80, an antibody that detects a determinant present on the majority of mouse macrophages, but not on fibroblasts (Austyn and Gordon, 1981; Hume et al. 1983). As can be seen in Fig. 3A, these cells do stain with F4/80, indicating that they are indeed of the macrophage lineage. Additionally, lysozyme RNA is readily detectable (data not shown).

In the presence of IL-3 alone, other myeloid lineages are not readily found in the EBs within the first 12 days of culture. At later stages (days 12 to 20), cells with the morphology of immature mast cells and to a lesser extent cells of the neutrophil lineage are observed amongst the differentiated progeny of the ES cells (Fig. 3C). To determine if mast cells could be expanded and maintained in culture, EBs at different stages were plated on to tissue-culture plates in the presence of IL-3. Upon transfer from methyl cellulose, EBs attach to the tissue-culture plastic and an adherent population of cells spread out. Within 5 to 10 days in the presence of IL-3, populations of mast cells appear. These mast cells appear to be similar in morphology to mast cells derived from adult marrow (Fig. 3D). In addition, with toluidine blue and May–Grünwald–Giemsa staining they show the characteristic granules of mast cells. This mast cell population can, in the presence of IL-3 be maintained and expanded for least ten weeks. In addition to mast cells, macrophages that were present after differentiation in methyl cellulose cultures can persist and continue to grow slowly.

These findings indicate that at least four separate hematopoietic lineages can be derived from *in vitro* differentiated ES cells in the presence of IL-3; i.e. erythroid, macrophage, mast cell and neutrophil. Furthermore, the sequential appearance of these lineages suggests that there is a predetermined developmental program within the EBs.

Effect of IL-1

IL-1 alone has no marked effect on the rate, or extent of erythroid development (data not shown). A moderate
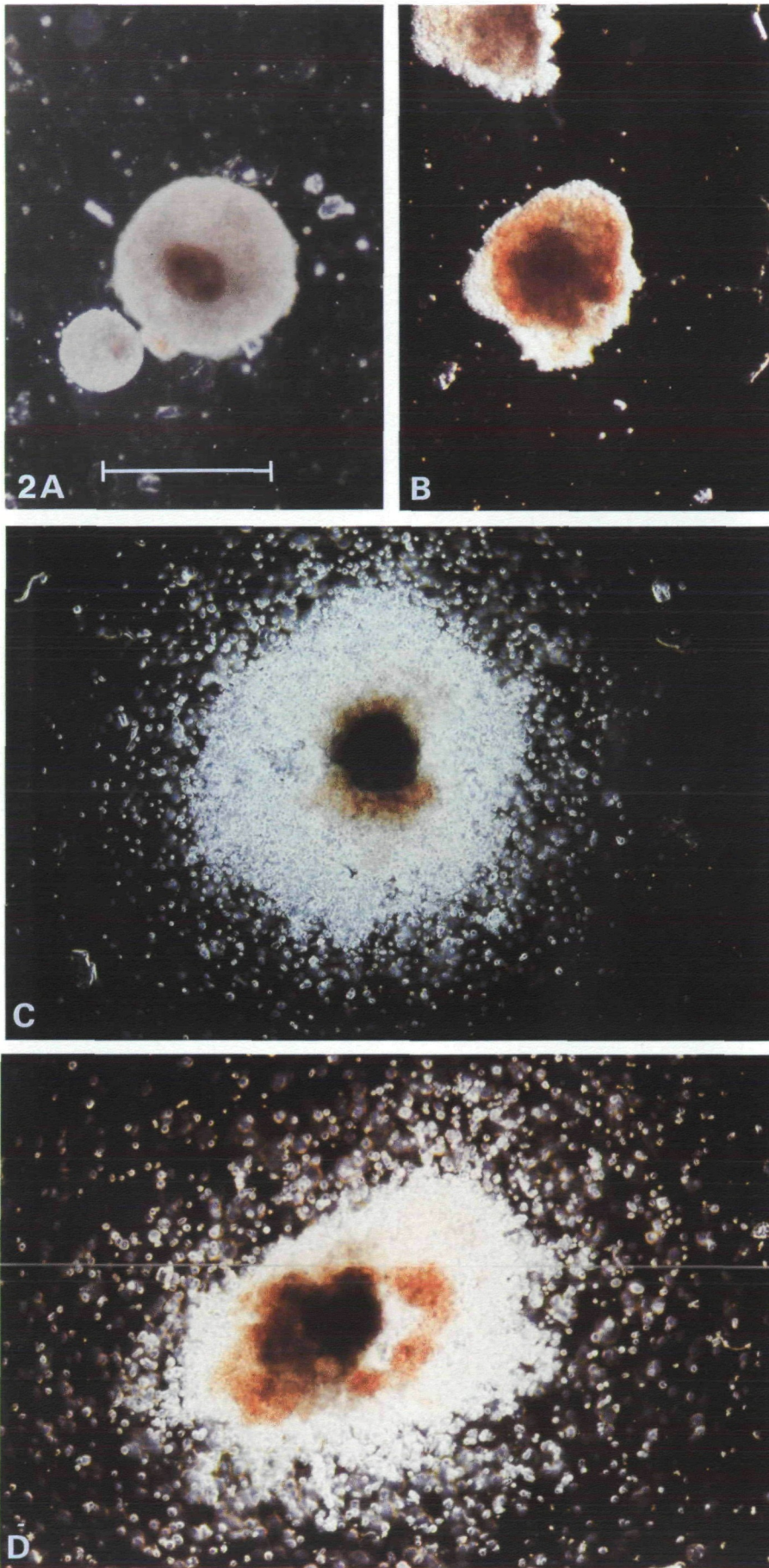
Fig. 2. EBs in methyl cellulose media, ±hematopoietic factors: bar=500 μm (A) EB grown in methyl cellulose media for 10 days with no additional factors; note area of globinisation in center of EB. (B) EB grown in methyl cellulose media for 12 days plus Epo; the EB has ruptured and a halo of nucleated red cells surround the original cell mass. (C) EB grown in methyl cellulose media for 13 days plus Epo, IL-1 and IL-3; the central cell mass is surrounded by a extending colony composed of nucleated erythrocytes, small macrophages, mast cells, a few neutrophils and other hematopoietic cells. (D) EB grown in methyl cellulose media for 15 days plus Epo, IL-1, IL-3 and M-CSF; the central cell mass is surrounded by nucleated erythrocytes and an extending mass composed almost entirely of large macrophages.
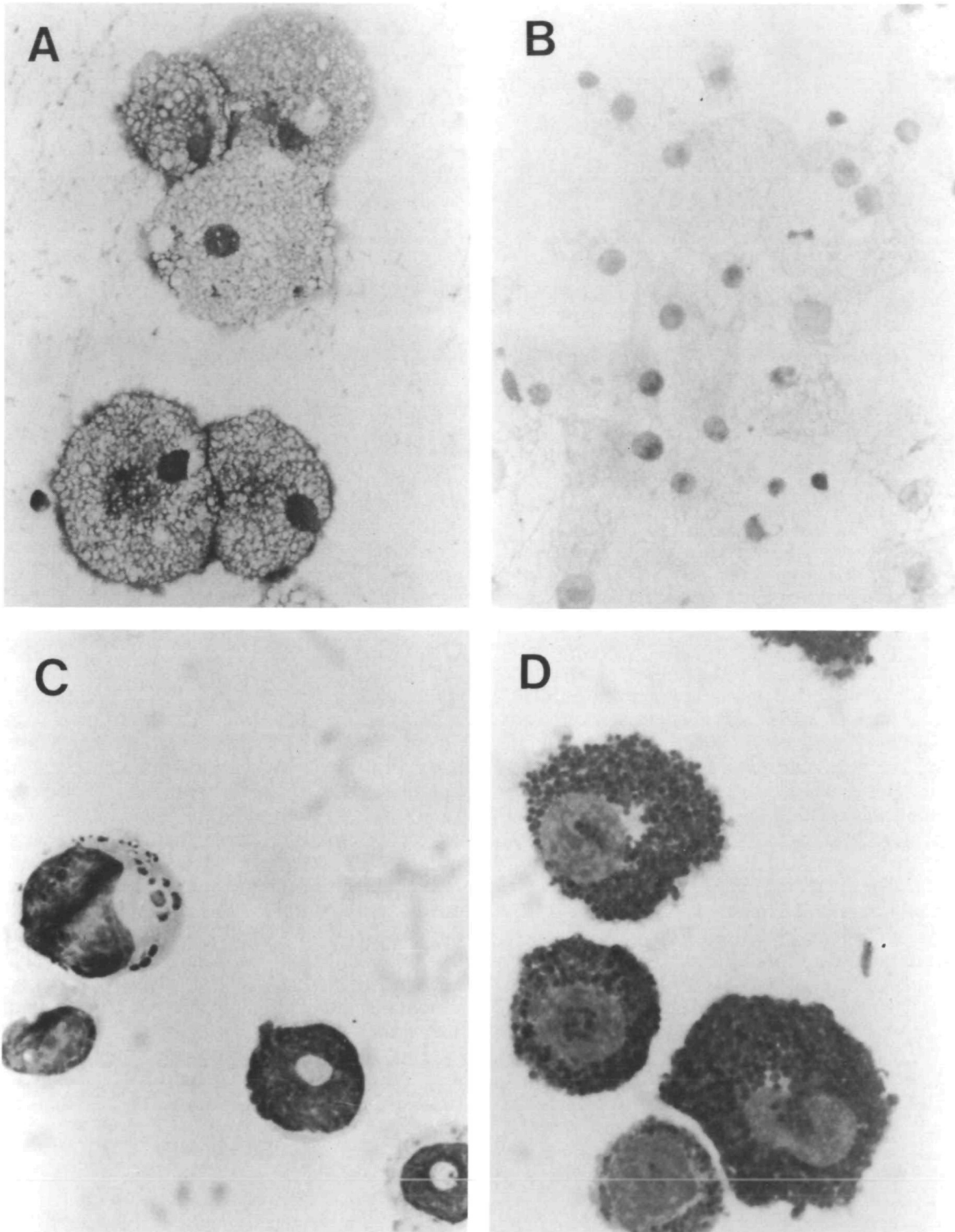
Fig. 3. Photomicrograph of hematopoietic cells from EBs (A,B) Cytospin of cells (×400) obtained from EBs grown in methyl cellulose media plus IL-3 for 15 days. Cells were stained with the macrophage-specific monoclonal F4/80. (A) With first and second antibody; (B) control with the second antibody alone. (C) Cytospin of neutrophils (×1000) obtained from EBs grown in methyl cellulose media plus IL-3 for 15 days, stained with May–Grünwald–Giemsa. (D) Cytospin of mast cells (×1000) stained with May–Grünwald–Giemsa showing characteristic staining granules. These cells were expanded out of EBs grown in methyl cellulose media plus IL-3 for 15 days and then transfered to liquid media plus IL-3. The cells could be harvested from the culture for more than 8 weeks.

increase in the persistence of redness is observed in association with EB-derived macrophages. The addition of IL-1 does, however, significantly increase the frequency of EBs that develop macrophages from 5–10% in the absence of factors to more than 30% after 15 days in culture (Fig. 1B).

Only a slight synergism in the development of erythroid cells is noted when IL-1 and Epo are combined. The combination of IL-1 and IL-3 gives rise to a higher frequency of EBs with macrophages and other hematopoietic cells than either factor alone (Fig. 2C). IL-1 has no dramatic effect upon the development of neutrophil or mast cell lineages.

Effects of GM-CSF and M-CSF

The addition of GM- or M-CSF has no marked effect on the rate and extent of erythroid development. As observed in cultures supplemented with IL-1, the decline of the visible erythroid component is somewhat delayed in EBs associated with macrophages. The percentage of EB colonies that develop macrophages increases to at least 20% in the presence of each of these factors. In addition, the macrophage component within the EBs is larger than that observed in the cultures containing IL-3 alone. When these CSFs are combined with IL-1 and/or IL-3, the frequency of EBs that contain macrophages increases further to at least 40%. Furthermore, these colonies contain many more cells, and can reach 2 to 3 mm in diameter. (Fig. 2D).

Taken together, these findings indicate that different growth factors can influence the further development of the various hematopoietic populations from differentiating EBs. The most effective combination of factors for the generation of the widest range of lineages is IL-3, IL-1 and Epo.

Globin expression in developing EBs

In the following series of experiments, we analyzed EBs grown under different conditions for the presence of embryonic and adult globin RNAs (βH1 and β^{maj}-globin). Embryonic z globin (βH1) is first detected at day 5 of differentiation (Fig. 4A). The level detected at this stage represents approximately 15% of the β-actin control. The RNA of β^{maj}-globin is not detected at day 5 (data not shown). By day 6 of differentiation, however, the adult β^{maj}-globin RNA is also found, the level being comparable to that of βH1 found at day 5 (Fig. 4B). The presence of Epo and/or IL-3 has no marked effect upon the timing or the expression level at this stage (Fig. 4A). The level of RNAs for both globins continues to increase until day 10 (data not shown). By day 12 the abundance of βH1 RNA drops rapidly, this loss is less marked in EBs cultured in the presence of Epo (Fig. 4A). The presence of IL-3 does not have any influence on the rate of loss of βH1 RNA.

The abundance of β^{maj}-globin RNA also begins to decrease by day 12 of culture; however, relative to the β-actin signal, this fall is less substantial than that of βH1. The fall in abundance of β-globin RNA is also less marked in the presence of Epo. IL-3 does not influence the abundance of β^{maj}-globin RNA (Fig. 4B). Compari-

son of the ratios of β^{maj}-globin to βH1 RNAs shows a shift from 0.04 at day 6 ($\pm$Epo and/or IL-3) to 0.24 by day 12, indicating a change in the relative proportions of these RNAs with time. These findings demonstrate that ES cells can rapidly differentiate to cells capable of producing both the embryonic and adult forms of globin.

Discussion

The data presented in this report are the first demonstration that ES cells can, at a high frequency differentiate into at least four distinct hematopoietic lineages when cultured under defined conditions.

Previous studies have shown that ES cells can generate primitive erythroid cells in culture (Doetschman *et al.* 1985). However, in order to obtain this population of differentiated cells, ES cells first had to be induced to form 'cystic embryoid bodies' and only after these structures developed could one identify small foci of erythroid cells. The appearance of these small foci was also largely dependent upon the presence of preselected batches of human cord serum. In addition, hematopoietic differentiation was considerably slower and unsynchronized.

It has been reported earlier that PCC3, an embryonal carcinoma (EC) cell originally derived from a transplantable tumor, can differentiate into hematopoietic cells in culture (Cudennec and Nicolas, 1977). To obtain blood islands, PCC3 cells had to be maintained in an organ culture system for approximately 10 days. However, the combined problems of the abnormal karyotype exhibited by EC cells and again the low level of erythropoiesis obtained limited the use of this system.

In contrast to the previous systems, when ES cells are plated directly into methyl cellulose media, they differentiate rapidly, in a synchronized manner. Additionally, they give rise to range of hematopoietic cells at a high frequency, in the absence of human cord serum and without prior formation of cystic bodies. We also find that with a given ES line, the kinetics of development of the hematopoietic population is highly reproducible. This enhanced hematopoietic activity observed in the semi-solid methyl cellulose system might be due to a local accumulation of factors in the methyl cellulose surrounding the EB. Such a local environment may help to induce and maintain a regime conducive to hematopoietic differentiation. This type of environment cannot form in the large volumes of media in a liquid culture suspension system.

EB development and embryogenesis

The development of hematopoietic cells within methyl cellulose cultures parallels to some extent the onset of hematopoiesis in the developing embryo. In the mouse embryo, erythropoiesis is first detected in the yolk sac at day 7.5 of gestation, with macrophages being present by day 10 of gestation (Moore and Metcalf, 1970; Cline and Moore, 1972). In addition, committed mast cell

precursors are identified as early as day 9.5 in the yolk sac and by day 11 in the fetal liver (Sonoda *et al.* 1983). In comparison, in methyl cellulose cultures, erythropoiesis is observed by days 7 to 8 of culture, while macrophages appear by day 10. The development of mast cell precursors is somewhat delayed in culture, appearing by day 12. If ES cells are regarded as similar to the inner cell mass of a 3.5-day blastocyst, then the appearance of hematopoietic cells from ES *in vitro* is approximately 3 to 4 days later than that observed with the embryo. The order of differentiation observed would indicate that the hematopoietic differentiation of ES cells *in vitro* is to some degree mimicking the *in vivo* situation, at least to the level of the yolk sac.
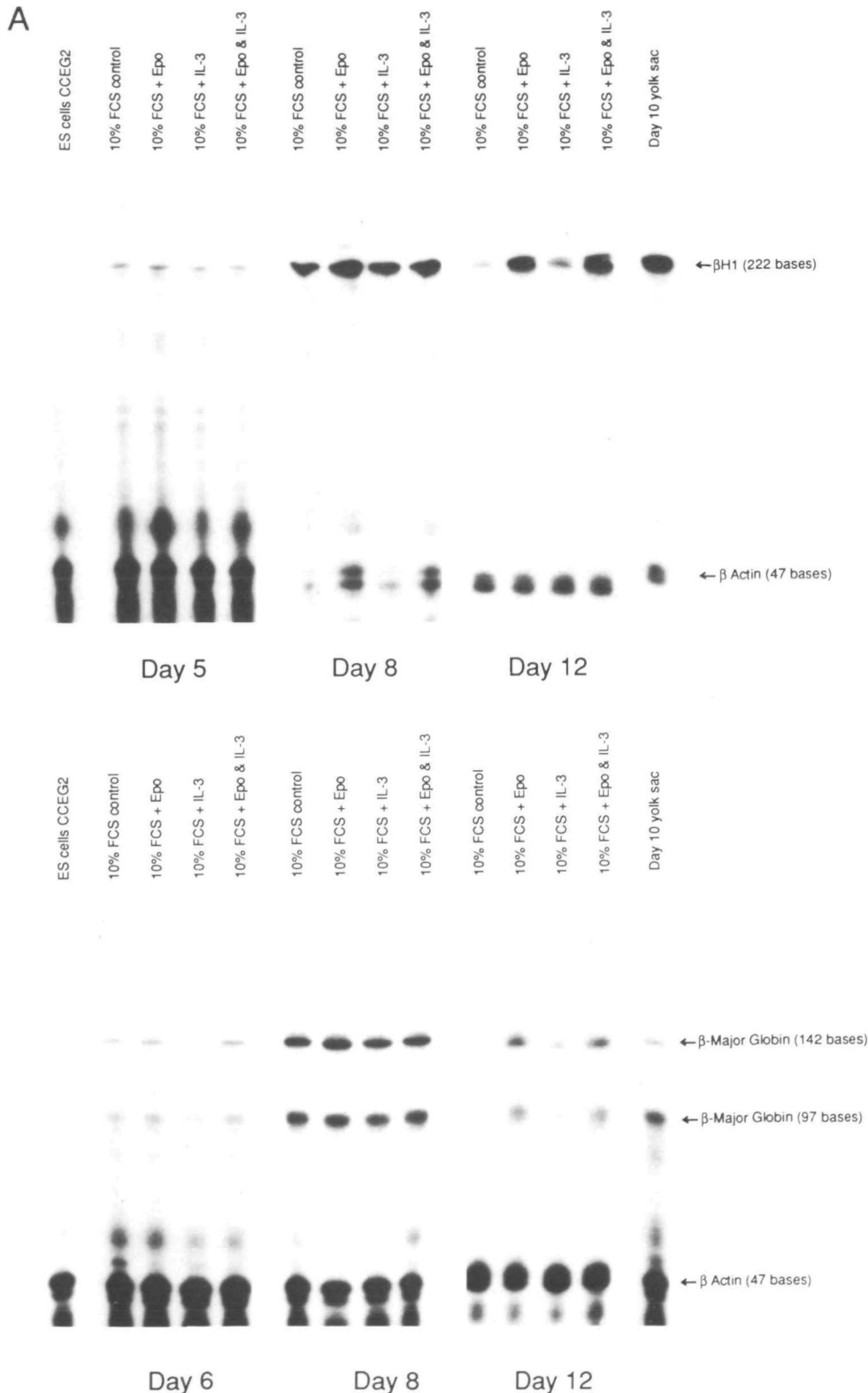
Fig. 4. RNAase protection on globin expression. RNAase protection assay using approximately 2 µg of total RNA. β-actin is used as an internal standard. Note, the exposures shown are aimed at showing the globin signal, as such the β-actin control tend to be over exposed. Signal intensities were quantified directly from the polyacrylamide gels using a 'Phosphor Imager' (Molecular Dynamics). (A) RNA derived from CCEG2 cells differentiated for 5, 8 and 12 days. Controls are undifferentiated CCEG2 and 10-day yolk sac derived RNAs. βH1 RNA when hybridized to the pBSBH1 RNA probe gives a protected fragment of 222 bases. When compared with the β-actin control, the signal for βH1 is similar for all four differentiation conditions on days 5 and 8; at day 12 the signal for βH1 is approximately three fold stronger in the presence of Epo, than for the other conditions. (B) RNA derived from CCEG2 cells differentiated for 6, 8 and 12 days. Controls are undifferentiated CCEG2 and 10 day yolk sac derived RNAs. β^{maj}-globin RNA when hybridized to the pBM700 RNA probe gives protected fragments of 142 bases and 97 bases. When compared with the β-actin control, the signal for β^{maj}-globin was similar for all four conditions on days 6 and 8; at day 12 the signal for β^{maj}-globin is approximately threefold stronger in the presence of Epo, than in the other conditions.

Globin synthesis in EBs

RNAase protection assays on RNA derived from CCEG2 differentiated under various regimes, first detects the embryonic globin βH1, at day 5 of differentiation while the adult β^{maj}-globin is first detected at day 6. Again this appears to mirror the developmental changes seen *in vivo*. Brotherton *et al.* (1979) and Wong *et al.* (1986) have shown that a population of large, nucleated erythrocytes are found in the yolk sac by day 9. Analysis of hemoglobin in these cells shows that there is a gradual shift in the ratio from predominantly embryonic to increasing amounts of adult hemoglobin with increasing time of gestation. In the differentiating EBs there is a shift in the ratio of β^{maj}-globin mRNA to βH1 from 0.04 at day 6, to 0.24 by day 12. The change that we observe may reflect a similar switching to that seen *in vivo*, or possibly a differential mRNA stability. It is interesting to note that this change in ratios occurs at the stage when Epo begins to exert a dramatic effect on the development of erythroid cells within the EBs. It is possible that Epo is causing the expansion of a population of cells that synthesize predominantly adult globin. Again this would correlate with the *in vivo* state (Wong *et al.* 1986).

In recent studies, we have seen a later wave (day 18) of erythropoiesis in cultures containing an increased amount of methyl cellulose media. The cells of this later erythroid population are significantly smaller than the early erythroid cells, growing in clusters reminiscent of colonies derived from adult erythroid precursors. It is possible that this later population are definitive erythroid cells, which would contain predominantly β^{maj}-globin. Cells from this later wave of erythropoiesis are currently being analyzed.

The effect of hematopoietic growth factors

The initial development of the erythroid and macrophage populations within the EBs is not dependent upon the addition of exogenous growth factors. This would suggest: (i) that some component/s of the culture system, for example FCS, contains low levels of factors that promote their development; (ii) that either these cells can develop in the absence of factors or that factors are produced within the developing EBs.

It is unlikely that the FCS contains significant amounts of hematopoietic growth factors, since at the concentrations used it is unable to simulate the growth of any precursors from normal marrow or support the growth of a *v-src* transformed erythroid cell line which responds to low levels of Epo (0.01 unit ml^{-1}) (G.K. unpublished observations). The most likely explanation is that EBs are programmed and/or are capable of producing factors that then drive their development. In support of hematopoietic factor production by EBs is the observation that significant amounts of IL-1, Epo and M-CSF mRNAs can be detected by PCR of cDNA derived from day 12 EBs (M.V.W. unpublished observations). Consistent with this interpretation is the detection of hematopoietic growth factors which stimulate the growth of macrophage and erythroid precursors

(CFU-E) and include M-CSF, an IL-3-like and an Epo-like activity in the embryo and the yolk sac fluid (Johnson and Metcalf, 1978; Johnson and Barker, 1985; Azoulay *et al.* 1987; Labastie *et al.* 1984). Although these findings are suggestive, the actual role of these or other factors in the embryonic development of hematopoietic cells still remains to be determined.

Development of EB-derived hematopoietic populations beyond day 10 of culture requires the presence of added factors. The role played by these factors is not currently known. It is possible they simply expand populations as they are generated during the development of the ES/EBs. Alternatively, they may actually influence the pathway along which the EB differentiates. The kinetics of the early lineages would indicate that initial ES differentiation is either preprogrammed or rapidly set by the local environment created within the developing EB and not influenced, at least in the early stages, by external factors.

The development of different myeloid lineages within the EBs suggests that multipotential stem cells can develop from ES cells *in vitro*. If significant numbers of these cells are generated, it should be possible to reconstitute the hematopoietic system of an irradiated recipient with differentiated EBs. In addition, if the 'correct' environment is developed, it should be possible to demonstrate the generation of lymphoid lineages from ES-derived multipotential cells. Experiments aimed at developing and identifying these populations are currently in progress. The success of these experiments will depend, to some extent on the developmental stage reached within the EBs. If we are essentially mimicking yolk sac development then the identification of the repopulating hematopoietic stem cell and lymphoid precursors might be difficult, as most studies indicate that the yolk sac contains relatively few of these cells (Moore and Metcalf, 1970; Paige, 1979; Toles *et al.* 1989). On the other hand, if we are able to achieve a stage of hematopoiesis representative of early fetal liver, the identification of primitive hematopoietic stem cells might be relatively easy, as these are plentiful in the fetal liver (Paige, 1983; Jordan *et al.* 1990)

In conclusion, the data presented here establish that ES cells can at a high frequency generate hematopoietic cells *in vitro*. The quantitative nature of this culture system provides a unique model for analyzing the effects of a wide spectrum of known and putative new growth factors on the earliest stages of hematopoietic development. In addition, the consistency with which hematopoietic cells develop in these cultures and the accessibility to large numbers of cells at various stages of differentiation provides a means with which to analyze the molecular events involved in the initial stages of hematopoietic commitment and development.

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