Transforming Growth Factor \( \beta_1 \) Is an Inducer of Erythroid Differentiation

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Summary

Normal human bone marrow cells, highly enriched for burst-forming units–erythroid (BFU-E), were cultured in serum-free medium, in the presence and absence of various factors, to investigate the mechanisms involved in regulating erythroid differentiation. In cultures containing interleukin 3 (IL-3), Steel factor (SF), and erythropoietin (Ep), benzidine-positive erythroblasts first became detectable on day 6. Their numbers then rapidly increased until, by day 16, >99% of the cells, which were 20,000-fold amplified over input numbers, were benzidine-positive. It is interesting to note that omission of either SF or Ep from this assay markedly enhanced the rate of differentiation and reduced total cell numbers, whereas omission of IL-3 had no effect on the rate of differentiation and only slightly reduced cell numbers. Of various agents tested, the most potent erythroid differentiation inducer (and inhibitor of cell proliferation) was found to be transforming growth factor \( \beta_1 \) (TGF-\( \beta_1 \)). This cytokine stimulated both the rapid appearance of hemoglobin-positive cells and an early cessation of cell proliferation. Using fluorescently tagged antibodies to glycophorin A and fluorescence-activated cell sorter (FACS) analysis, this phenomenon was shown to be due to an early induction of erythroid differentiation rather than an aberrant production of hemoglobin. Methylcellulose assays indicated that the well-documented reduction of BFU-E colony numbers observed with TGF-\( \beta_1 \) may actually be due to a TGF-\( \beta_1 \)-induced "conversion" of BFU-E into colony-forming units–erythroid (CFU-E). Thus, in vivo, TGF-\( \beta_1 \) might serve, in part, to decrease the number of mature erythrocytes by stimulating BFU-E to skip a number of cell divisions and differentiate early.

The intracellular mechanisms that regulate the proliferation and differentiation of primitive erythroid progenitor cells are still largely unknown. This has been due, in large part, to the difficulties inherent in obtaining sufficiently large, homogeneous starting populations of primitive erythroid progenitors for biochemical analysis and the difficulties in growing these cells under well-defined serum-free conditions. However, with recent improvements in cell sorting techniques (1) and with the optimization of growth conditions using readily available recombinant growth factors (1, 2), such studies have now become feasible. In this report we present our findings using an erythroid differentiation assay in which CD34\(^+\)CD71\(^hi\)CD45RA\(^-\) human bone marrow cells, purified by FACS\(^*\), were allowed to proliferate and differentiate into hemoglobin containing cells under serum-free conditions in suspension cultures containing IL-3, Steel factor (SF)\(^1\), erythropoietin (Ep), insulin, BSA, and iron-saturated transferrin. Under the conditions used, cell numbers increased dramatically, plateauing at 20,000-fold amplification by day 16 of incubation. Moreover, by day 12, >99% of the cells were benzidine-positive. This assay system has allowed us to investigate the consequences of various growth factor additions and withdrawals on the proliferation and differentiation of burst-forming units–erythroid (BFU-E). Our results suggest that SF and Ep may function in vivo to delay the erythroid differentiation of mature BFU-E and thus allow more cell divisions and increased numbers of mature red blood cells. TGF-\( \beta_1 \), on the other hand, may perform the opposite function by prematurely triggering terminal erythroid differentiation in primitive erythroid progenitors and thereby reducing the overall number of mature erythrocytes.

Materials and Methods

Reagents. TGF-\( \beta_1 \), TNF-\( \alpha \), and macrophage inflammatory protein 1 \( \alpha \) (MIP-1\( \alpha \)) were purchased from R & D Systems Inc. (Minneapolis, MN). Recombinant human activin was a generous gift...
from Genentech Inc. (San Francisco, CA), and recombinant human SF was provided by Amgen Biologicals (Thousand Oaks, CA). Recombinant human IL-9 and IL-11 were from Genetics Institute (Cambridge, MA). PIXY was generously provided by Dr. D. E. Williams of Immunex (Seattle, WA). BSA (Fraction V, A4503), prostaglandin E₁ and E₂, human glucagon, human somatostatin (growth hormone [GH]), retinoic acid, spermidine, thymidine, hexamethylene bisacetamide (HMBA), and low density lipoproteins (L2139) were all purchased from Sigma Chemical Co. (St. Louis, MO). DMso was obtained from Fisher Scientific (Vancouver, BC, Canada). Recombinant human Ep and IL-3 were purified from culture supernatants of baby hamster kidney cells expressing an Ep cDNA and COS cells transfected with human IL-3 cDNA, respectively, as described previously (3). Leukemia inhibitory factor (LIF) were generously provided as a COS cell supernatant from Dr. D. Metcalf (Walter and Eliza Hall Institute, Melbourne, Australia). 

Monoclonal Antibodies. Mouse monoclonal IgG₁ antibodies specific for CD34 (8G12), CD71 (OKT9), CD45RA (8d2), and glycophorin A (10F7MN) were purified from hybridoma tissue culture supernatants using protein A or protein G affinity chromatography and labeled with cyanin 5-succinimidylester (Cy-5), FITC and R-phycocerythrin (PE) (for both 8d2 and 10F7MN), respectively, as described previously (1).

Purification of Human BFU-E. Haptenized bone marrow was obtained from informed and consenting normal individuals donating marrow for allogeneic transplantation or from vertebral bodies of cadaveric organ donors. Low-density cells (<1.077 g/cc) were isolated using Ficoll-Paque (Pharmacia LKB, Upppsala, Sweden), resuspended in Iscove's medium with 50% FCS and DNase 1 (0.1 mg/ml) (Cat. no. D4513; Sigma Chemical Co.), and then washed once and resuspended in Hank's Hapes-buffered salt solution containing 2% FCS, 0.1% sodium azide (HFN), 0.1 mg/ml DNase 1, and 5% normal human serum and incubated at 10⁶ cells/ml simultaneously with 8G12-Cy5, 8d2-PE, and OKT9-FITC at 10, 4, and 1 μg/ml, respectively, for 30 min at 4°C. The cells were then washed twice in HFN and resuspended in HF containing propidium iodide (Cat. no. p-4170; Sigma Chemical Co.) and sorted on a FACStar® (Becton Dickinson & Co., Mountain View, CA) as described previously (1). The sorted cells were either used directly or aliquoted (20,000 cells/vial), frozen in Iscove’s medium containing 2.5% human serum albumin and 7.5% DMso, and stored at -70°C.

Serum-free Suspension Cultures. On day 0, fresh or frozen CD34⁺/CD71⁺/CD45RA⁺ cells were washed with Iscove’s MEM, resuspended in serum-free medium (2% BSA, 10 μg/ml insulin, 200 μg/ml iron-saturated transferrin, 40 μg/ml low density lipoproteins, 5 x 10⁻⁸ M-2-mercaptoethanol, and penicillin/streptomycin at 10² U and 50 μg/ml, respectively) containing IL-3 (20 ng/ml), SF (50 ng/ml), and Ep (1 U/ml) (1), unless otherwise indicated, and 500–5,000 cells/well incubated in 96-well flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark) at 37°C in a water saturated atmosphere of 5% CO₂ for the next 16 d. The number of viable cells, using trypan blue, and the number of benzidine positive cells (4) were determined over this 16-d period.

Assay for Clonogenic Cells. Colony assays for erythroid (CFU-E, BFU-E), granulocyte/macrophage (CFU-GM), and multipotent CFU-granulocyte/erythroid/megakaryocyte/monocyte (CFU-GEMM) progenitors were performed in methylcellulose cultures as previously described (5), using MethoCult H4431 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 20 ng/ml PIXY, and 50 ng/ml SF, unless otherwise indicated.

Results

As a first step towards elucidating the regulatory mechanisms that control normal human erythropoiesis, a simple serum-free suspension assay was devised to monitor both the proliferation and differentiation of BFU-E from normal human bone marrow. For this, low density cells, obtained by Ficoll-Paque centrifugation, were fractionated by FACS®, using monoclonal antibodies to CD34, the transferrin receptor (CD71), and CD45RA, as described in Materials and Methods, and the selected cells, shown in Fig. 1 were then used directly or aliquoted at 20,000 cells/vial, frozen and stored at -70°C. Typically, for the assay, 500–5,000 cells, in a final volume of 0.2 ml, were added per well to 96-well flat-bottomed tissue culture plates containing IL-3, SF, and Ep in a serum-free medium previously selected for its ability to maximally stimulate the proliferation of CD34⁺ cells in suspension cultures (1). As can be seen in Fig. 2 A, these culture conditions allowed for the rapid proliferation of these cells during the next 16 d at 37°C, at which time they plateaued at an ~20,000-fold amplification of starting cell numbers. Monitoring the number of benzidine-positive cells from these same cultures during this time period revealed that cells containing hemoglobin first appeared on day 6 and their numbers increased steadily until, by day 12, >99% of the cells were benzidine-positive (Fig. 2 B). This rate of hemoglobinization was unaffected by the number of cells used to initiate the cultures, at least in the 500–5,000 cell range tested (data not shown).

We then set out to determine the effect of omitting one or more of the three hemopoietic cytokines that were routinely added to these cultures (Fig. 3). It is interesting to note that leaving out SF decreased total cell numbers and markedly enhanced the rate of erythroid differentiation of these cells. Also of interest was the finding that normal human BFU-E were capable of giving rise to hemoglobin-positive cells in the absence of Ep and actually did so at a substantially faster rate than in its presence. To ensure that these cells were not becoming hemoglobin-positive because of a low level of endogenously produced Ep, these cultures were also...
tested in the presence of an excess of neutralizing anti-Ep 26 monoclonal antibodies (6) (Fig. 3). As with SF, total cell numbers were reduced, but even more so when SF was omitted, consistent with the antiapoptotic role recently reported for Ep (7–9). Omitting IL-3 resulted in a slight decrease in total cell numbers without significantly affecting the rate of erythroid differentiation. Adding three times the usual amount of IL-3, SF, and Ep to these cultures increased the total cell counts two to threefold but had no effect on their rate of differentiation (data not shown).

We next examined the effect of various factors that, from the literature, might be expected to have an influence in our assay system. These included TGF-β1, MIP-1α, and glucagon; which have been shown previously to inhibit erythropoiesis under certain conditions (10–16); activin, human somatotropin (GH), prostaglandins E1 and E2, LIF, human IL-9, and IL-11, all of which have been reported to increase erythroid colony numbers in methylcellulose (17–23); and TNF-α, activin, TGF-β1, retinoic acid, spermidine, HMBA, DMSO, and the cell cycle blockers thymidine, aphidicolin, and 5-azacytidine, all of which have been shown to induce differentiation in some systems (24–40). We tested these reagents over a wide range of concentrations and the results, shown in Table 1, are those obtained with concentrations that gave the most pronounced effects on differentiation. Agents that increased the rate of differentiation, and concomitantly decreased total cell numbers in this suspension assay included activin A, the cell cycle blockers, thymidine and aphidicolin, and the MEL cell differentiation inducing agents, DMSO and HMBA. How-
Table 1. Effect of Various Agents on the Proliferation and Differentiation of CD34+CD71hiCD45RA- Cells

| Experimental* | Control† |
|---------------|---------|
| MIP-1α (500 ng/ml) | 11% (6.0 X 10⁶) | 13% (6.0 X 10⁶) |
| TGF-β1 (20 ng/ml)  | 92% (3.0 X 10⁶) | 18% (1.0 X 10⁶) |
| Activin A (120 ng/ml) | 29% (1.1 X 10⁶) | 17% (1.1 X 10⁷) |
| Aphidicolin (5 µg/ml) | 59% (3.2 X 10⁷) | 13% (1.2 X 10⁷) |
| 5-azacytidine (0.5 mM) | 12% (5.7 X 10⁷) | 15% (5.4 X 10⁷) |
| Thymidine (100 µM) | 52% (1.6 X 10⁴) | 22% (1.3 X 10⁴) |
| Glucagon (100 µg/ml) | 18% (1.0 X 10⁶) | 18% (1.0 X 10⁶) |
| HMBA (2.5 mM) | 30% (1.1 X 10⁴) | 13% (6.0 X 10⁴) |
| DMSO (1%) | 30% (1.1 X 10⁴) | 13% (6.0 X 10⁴) |
| hGH (100 ng/ml) | 10% (1.0 X 10⁴) | 13% (6.0 X 10⁴) |
| PGE₁ (100 nM) | 35% (1.2 X 10⁴) | 37% (1.3 X 10⁴) |
| PGE₂ (100 nM) | 43% (7.8 X 10⁴) | 37% (1.3 X 10⁴) |
| LIF (100 ng/ml) | 17% (8.0 X 10⁴) | 13% (6.0 X 10⁴) |
| hIL-9 (100 ng/ml) | 9% (7.0 X 10⁴) | 13% (6.0 X 10⁴) |
| hIL-11 (100 ng/ml) | 33% (1.4 X 10⁴) | 37% (1.3 X 10⁴) |
| Retinoic acid (2 µM) | 20% (1.1 X 10⁴) | 13% (6.0 X 10⁴) |
| Spermidine (3 nM) | 19% (0.7 X 10⁴) | 26% (2.0 X 10⁴) |
| TNF-α (50 ng/ml) | 37% (5.2 X 10⁴) | 37% (1.3 X 10⁴) |

* Percent refers to percent of benzidine positive cells on day 7. Numbers in parentheses refer to total cell number on day 7.
† Percent and numbers in parentheses refer to benzidine positive and total cell numbers, respectively, of control cultures obtained on day 7 within the same experiment.
§ Added to cultures on day 4.
¶ These assays were initiated with 500 cells/well, the rest were started with 5,000 cells/well.

ever, the most potent agent in this regard was the growth factor, TGF-β1 (Table 1). We therefore set out to examine the effect of this cytokine more closely in our assay system.

A TGF-β1 dose-response analysis was carried out and revealed that as little as 0.4 ng/ml significantly reduced the time required for 50% of the cells to become hemoglobin-positive (Fig. 4). At 4 ng/ml of TGF-β1, >50% of the cells were hemoglobin-positive by day 4. Similar numbers of hemoglobinizing cells were not observed in control cultures, containing SF, IL-3, and Ep, until day 9 (Fig. 4 A). At 40 ng/ml of TGF-β1, >50% of the cells were hemoglobin-positive by day 4. Similar numbers of hemoglobinizing cells were not observed in control cultures, containing SF, IL-3, and Ep, until day 9 (Fig. 4 A). At 40 ng/ml of TGF-β1, >50% of the cells were hemoglobin-positive by day 4. At 40 ng/ml of TGF-β1, >50% of the cells were hemoglobin-positive by day 4. Also, as expected, an inverse correlation was observed between TGF-β1 concentration and total cell output, with TGF-β1 causing a plateau in cell numbers earlier than in control cultures (Fig. 4 B). These studies also revealed that TGF-β1 stimulated an increase in the absolute number (and not just the percent) of benzidine positive cells (Fig. 4 C), consistent with the notion that this cytokine is stimulating an increased rate of erythroid differentiation and not simply a growth inhibition of more primitive erythroid progenitors in the cell population.

A delayed addition study was then carried out with TGF-β1 to gain some insight into the target cell for the effects observed here. As can be seen in Fig. 5, TGF-β1 stimulated differentiation and started inhibiting total cell output within 24 h of addition, regardless of when it was added. This suggests that most if not all erythroid progenitors, from BFU-E onward, are target cells for TGF-β1.

Many reports in the literature have suggested that TGF-β1 inhibits BFU-E formation (41-45). From our suspension culture data, it appeared possible that TGF-β1 might be inhibiting BFU-E proliferation without blocking differentiation, thus effectively “converting” BFU-E into CFU-E. To test this, CD34+CD71hiCD45RA- cells were cultured in complete methylcellulose medium to allow the clonal progeny of each initial cell to be evaluated over time. As can be seen in Table 2, the overall plating efficiency of the cells in control cultures containing IL-3, SF, and Ep, was the same as cultures containing TGF-β1 (i.e., 50-60%). In the presence of TGF-β1, however, there was a substantial decrease in the number of large erythroid colonies (normally scored as BFU-E derived) and a concomitant increase in the number of small erythroid colonies (normally scored as CFU-E derived). It is interesting to note that the total number of erythroid colony...
numbers was not affected by the presence of TGF-β1, confirming that TGF-β1 was not giving the appearance of speeding up hemoglobinization by simply inhibiting the growth of more immature erythroid progenitors.

To confirm that TGF-β1 was indeed inducing the erythroid differentiation of CD34⁺CD71hiCD45RA⁻ cells and not just turning on hemoglobin synthesis and also to more accurately quantitate the absolute numbers of cells differentiating in the presence and absence of TGF-β1, a second marker of erythroid differentiation, glycophorin A, was examined by FACS® analysis. A time course revealed that, by day 2, TGF-β1 had dramatically increased the absolute number of glycophorin A⁺ cells, even though total cell numbers were decreased (Fig. 6). It is interesting to note that in control cultures, the majority of differentiating cells lost CD34 from their surfaces before expressing glycophorin. In contrast, when cells were incubated with TGF-β1, the bulk of glycophorin A⁺ cells were also CD34⁺, perhaps reflecting the fewer cell divisions these cells go through in the presence of this cytokine. Consistent with this notion, the total number of cells plateaued on day 7 in the presence of TGF-β1 while control cultures continued to increase their total cell numbers until day 12 (data not shown).

Discussion
In this report we describe the use of a 16-d erythroid differentiation assay to examine the effects of various agents on the proliferation and differentiation of normal human BFU-E. Under the standard culture conditions of this assay, the cells differentiate in a synchronous fashion and cell proliferation plateaus at an ~20,000-fold amplification which suggests, given a 50–60% plating efficiency, that these cells divide approximately 16 times before arresting (assuming minimal cell death). Omission of SF or Ep from these cultures enhances differentiation and reduces the proliferation potential of the starting cell population to approximately 10 divisions (again assuming minimal cell death, which is overly simplistic, given the antiapoptotic properties of these cytokines [7–9, 46]). This could suggest that, in vivo, both SF and Ep function to delay the onset of differentiation, thus allowing more cell divisions and an increase in the numbers of mature red blood cells. In the case of SF, this is consistent with a very recent report by Ogawa et al. (47), who showed that SF inhibits the erythroid differentiation of K562 cells. Although postulating such a function for Ep may be somewhat heretical it is supported to some extent by very recent experiments using an MEL cell line transfected with a temperature-sensitive mutant of p53 (48). At the permissive temperature these cells arrest in the Go/G1 phase of the cell cycle, rapidly lose viability and express hemoglobin. However, when Ep positive (A) and total cell numbers (B) determined daily. C is a replotting of the data for the control (■) and 40 ng/ml TGF-β1 (▲) cultures to indicate the total number of benzidine positive cells. Each point represents the mean ± SEM of duplicate samples. In this experiment, cultures were initiated with 5,000 cells/well.
Table 2. Effect of TGF-β1 on Colony Formation from CD34+CD71+CD45RA- Cells

| Addition          | CFU-E | Mature BFU-E | Immature BFU-E | CFU-GEMM | CFU-GM | Total Colonies |
|-------------------|-------|--------------|----------------|----------|--------|----------------|
| Control           | 10 ± 2| 55 ± 2       | 38 ± 4         | 6 ± 2    | 3 ± 1  | 112 ± 10       |
| TGF-β1 (20 ng/ml) | 92 ± 4| 12 ± 2       | 0              | 1 ± 1    | 1 ± 1  | 106 ± 8        |

* Methycellulose assays were performed using 200 cells/dish in MethoCult H4431 (i.e., 30% FCS, 10% agar leukocyte conditioned medium, 1% BSA, 10-4M 2-mercaptoethanol, 3 U/ml Ep, and 2 mM glutamine) supplemented with 20 ng/ml PIXY and 50 ng/ml SF. Similar results were obtained in three separate experiments.

is added to these cultures it prolongs their survival and diminishes the extent of hemoglobin production. It is therefore conceivable that Ep delays the terminal erythroid differentiation program of primitive progenitors (i.e., mature BFU-E and CFU-E) while promoting this same program in more mature precursors (i.e., proerythroblasts and normoblasts).

Of the various agents added to this assay, DMSO and HMBA, two agents that have been shown previously to stimu-
late the differentiation of Friend virus-transformed murine erythroid cells (i.e., MEL cells) were found to significantly increase the rate of differentiation and concomitantly decrease the rate of proliferation of normal human erythroid progenitors. This not only adds further credibility to the use of the ubiquitous cytokine is produced by most normal tissues (49) i.e., they may stimulate the release of factors by accessory cells E2, LIF, IL-9, IL-11, retinoic acid, spermidine, and TNF-α. Based on our data and results obtained from other studies it is likely these agents were without effect because their target cells are not BFU-E or that they do not act directly on BFU-E; i.e., they may stimulate the release of factors by accessory cells that affect BFU-E differentiation.

It is interesting to note that of the various agents added to this assay, TGF-β1 gave the most profound effects. This ubiquitous cytokine is produced by most normal tissues (49) and virally transformed cells (50, 51) and has been reported to act as a chemotactic factor (52), an enhancer of integrin expression and type IV collagenase secretion in monocytes (53), a mitogen (54), a growth inhibitor (55, 56), a differentiation inhibitor (57), and a differentiation inducer (58, 59). It is found at high concentrations in the centers of active tissue differentiation, including bone marrow and areas containing hemopoietic stem cells in fetal liver (60), consistent with its putative in vivo role as a differentiation inducer. In both human and murine primary bone marrow cell cultures, TGF-β has been reported to inhibit both multilineage colony formation (CFU-GEMM) and BFU-E, while stimulating CFU-E (43, 44, 51). Our delayed addition and methylcellulose assay results with TGF-β1 suggest that this is not due to an inhibition of BFU-E growth and a stimulation of CFU-E numbers but rather to a conversion of BFU-E into CFU-E. This is further supported by the earlier cessation of cell proliferation (i.e., plateauing of cell numbers) observed in the presence of TGF-β1 (see Figs. 4 and 5). It is interesting to note that studies carried out with the multipotent human erythroleukemic cell line, K562, have shown that TGF-β1, and to a lesser extent, activin, could induce hemoglobin accumulation and a concomitant reduction in proliferation and cell size (23), reminiscent of the effects we report herein with normal human bone marrow-derived BFU-E. Other studies using K562 cells have revealed that it can also be induced to hemoglobinize with agents that halt cell division, like 5-aza-cytidine (62), adriamycin (63), arabinofuranosylcytosine (64), and aphidicolin (37). The ability of TGF-β to induce erythroid differentiation in this cell line thus may depend upon its ability to inhibit cells from entering S phase, and there is a growing consensus that TGF-β mediates its effects on target cells by arresting or delaying cells in G1 of the cell cycle. This is consistent with our finding that two agents that normally inhibit entry into S, i.e., thymidine and aphidicolin, also stimulated hemoglobinization in our assay. The mechanism by which TGF-β1 inhibits erythropoietic proliferation is not completely understood although there is some evidence to suggest that it may do so, at least in part, by maintaining the retinoblastoma (Rb) growth suppressor protein in an active, underphosphorylated state (65, 66). In fact, very recent results with human keratinocytes and mink lung epithelial cells suggest that it may do this by either inhibiting the synthesis of the cyclin dependent kinase (and/or its associated cyclin) that phosphorylates Rb (67, 68) or by blocking the formation of this cyclin-kinase complex (69). An alternative to this model is that TGF-β1 may inhibit cell cycling by downmodulating the expression of growth factor receptors (70). For example, downmodulation of IL-1 receptors by TGF-β has been proposed as a mechanism of TGF-β-induced inhibition of IL-1 action (71). TGF-β has also been reported to modulate the expression of GM-CSF, IL-3, and CSF-1 receptor numbers (71–73) and has recently been shown to interfere with the proliferation-inducing activity of SF in myelogenous blasts, possibly through functional downregulation of the c-kit protooncogene (71). Since we observed a profound erythroid differentiating effect when SF was omitted from our assay medium, and a drop in total cell numbers equal to that obtained with TGF-β1, it is conceivable that TGF-β1 stimulates BFU-E differentiation by downmodulating SF receptors. We are currently investigating whether effects on suppressor protein phosphorylation and/or receptor modulation are involved in the action of TGF-β1 on the immediate progeny of normal human BFU-E.

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