An Endogenous Signal Triggering Erythroid Differentiation: Identification as Thyroid Hormone

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Abstract

The identification of thyroid hormone as an endogenous signal for erythroid differentiation began with our studies on the spontaneously differentiating murine erythroleukaemia clone 3–1. We observed that the spontaneous differentiation frequency was dependent on a heat stable factor present in fetal calf serum or calf bone marrow. We also noted that the bone marrow extract stimulated erythroid colony-forming units in mouse bone marrow cells, suggesting the relevance of this factor in normal erythroid differentiation. The bone marrow extract did not supplant the requirement of erythropoietin but was synergistic. Purification of the bone marrow extract indicated that the differentiation-inducing activity for clone 3–1 cells cochromatographed with a low-molecular-weight, UV (280 nm)-absorbing component(s). These observations and previous reports identifying the avian erythroblastosis virus oncogene v-erbA as a mutated thyroid hormone receptor which blocked erythroid differentiation led us to test thyroid hormone in our assay. Both triiodothyronine and thyroxine were highly active, and the active constituents in the chromatographically pure fraction were identified as triiodothyronine and thyroxine. Although thyroid hormone action has been associated with both in vivo and in vitro erythroid differentiation, its role has been often relegated to a secondary status. We suggest that thyroid hormone is required for the commitment of erythroid cells to terminal differentiation.

Introduction

The wide-ranging effects of TH raise the possibility that TH may have multiple roles in the development of the muscle, skeletal, and central nervous systems as well as such functions as metabolism and calorigenesis. The role of TH on developmental processes has often been considered to be indirect and to involve the modulating action of other hormones. For example, in a reference to TH action in the tadpole, it has been suggested that "TH does not initiate events, but rather regulates rates" (3). In another example, TH action in erythroid differentiation has been attributed to the increased production of EPO or hematopoietic factors by nonerythroid cells (4).

The recent interest in the role of TH receptor and by implication TH in differentiation has been primarily focused on the avian system where one of the two avian erythroblastosis virus oncogenes, v-erbA, had been identified as a mutated version of the TH receptor (5, 6). In subsequent studies, it was revealed that v-erbA blocked erythroid differentiation (7), apparently at the CFU-E stage (8) and arrested the transcription of band 3, a major anion transporter in erythroid cells, 5-aminolevulinic acid synthetase, an enzyme involved in heme biosynthesis and carbonic anhydrase II (9, 10). In addition, the overexpression of the TH(T3) receptor as a gag-c-erbA fusion protein enhanced erythroid differentiation in a T3-dependent fashion (11). It was also noted in these studies that the endogenous TH receptor did not confer a detectable T3 response in the differentiation of erythroblasts. In other studies, it was shown that the chicken c-erbA protooncogene was preferentially expressed during the late stages of erythroid differentiation (12). Schroeder et al. (13) observed that RA and/or TH slightly accelerated the differentiation of immature erythroid progenitor cells, and caused premature death for more mature cells. They suggested that RA and/or TH modulate rather than trigger erythroid differentiation. In studies using transforming growth factor-α-induced embryonic bone marrow cells, Gandrillon et al. (14) have proposed a model of the erythrocytic pathway in which RA or T3 functions in the precommitment phase as "apoptotic-inducing" factors, and EPO plus insulin functions subsequently as differentiation-inducing factors. Whereas the avian studies clearly indicated the perturbing influence of v-erbA on erythroid differentiation, physiological roles for the endogenous c-erbA, TH, or RA remains uncertain.

The studies reported here mainly concern the identification of TH as an endogenous inducer of erythroid differentiation. These studies began with the objective of identifying the endogenous signal that triggered the spontaneous differentiation of murine FEL clone 3–1 cells (15). We observed that this spontaneous differentiation was dependent on a partially dialysable, heat stable component found in FCS or CBM. Purification of this activity from CBM revealed that the differentiation-inducing factor cochromatographed with a UV (280 nm)-absorbing, low-molecular-weight constituent(s). In subsequent studies, we have identified this differentiation-inducing activity as TH. Whereas our studies were mainly carried out with the neoplastic FEL cell line,
the generality concerning the role of TH in erythroid differentiation is evident and will be discussed.

Results

Preliminary Studies. FEL cells spontaneously differentiate at frequencies generally <1% (15). We had noted previously that this spontaneous differentiation was dependent on the presence of FCS in the growth medium and that calf serum which supported comparable levels of growth was largely ineffective in promoting differentiation. The present studies, which began with our studies of clone 3–1, corroborated our earlier observations and also showed that the activity was heat stable (as shown later) and that much of the activity in FCS was lost on dialysis. The differentiation frequency with undialyzed and dFCS was 9.2 ± 1.0% and 2.2 ± 0.5%, respectively. Another source of FCS (Hyclone, Logan, UT) also showed levels of activity comparable to that of undialyzed serum. The parental (745A) cells also responded similarly to dialyzed and undialyzed FCS but differentiated at a much reduced level (Fig. 1, e and f). Clone 3–1 cells grown in medium containing “Nu serum” showed very depressed levels of differentiation (1.0 ± 0.2%). These observations made with Nu serum and dFCS formed the basis of the assay for the quantitation of the differentiation-inducing activity (see “Materials and Methods” and Fig. 2). Clone 3–1 cells were indispensable for the assay since these cells provided an amplified response to the differentiation-inducing factor. However, the clone was somewhat unstable, and the “control values” using dialyzed and undialyzed FCS decreased with cultivation. Consequently, the cells were recloned every 2 months.

Prior to initiating a purification program, we conducted a preliminary characterization of the differentiation-inducing activity. Extracts prepared (as described below) from CBM, spleen, and thymus showed activity, whereas liver and kidney showed no detectable activity (Table 1). We also observed that extracts prepared from CBM stimulated the differentiation of erythroid cells derived from mouse bone marrow (Fig. 3). The development of these erythroid colonies (CFU-E) required EPO as previously shown (16). The extract had little or no discernible effect by itself but had a clear synergistic effect when combined with EPO. These results indicate that a factor (or factors) present in the bone marrow extract also stimulated differentiation of bone marrow-derived erythroid cells at the CFU-E stage. Although the clone 3–1 and mouse bone marrow assays are very different in character, it is, nevertheless, interesting to note that the quantity of extract required for one-half maximal stimulation in both assays are similar (Figs. 2 and 3).

In these preliminary studies, FCS and tissue homogenates were usually fractionated using four volumes of 95% ethanol (pH 4.0). The presence of activity in the supernatant fraction (“extract”) suggested that the active component(s) was not a typical protein. Chromatography of the FCS-derived extract by gel filtration (Bio-Gel P-10) using 0.3 M

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4 K. Ebisuzaki, unpublished observations.
acetic acid for elution indicated a heterogeneous, somewhat variable distribution of activities ranging in estimated molecular weights (M, >1,000 to >10,000; Fig. 4A). This observation suggested the presence of several different activities or of a single activity bound to heterogeneously fragmented peptides. When CBM was homogenized in ice-cold phosphate buffer (0.01 M, pH 7.4), alcohol fractionated and chromatographed as described above, there was a similar heterogeneous distribution of activities (data not shown). Since we suspected that the heterogeneity observed on gel filtration chromatography might be due to proteolysis, the homogenization of bone marrow was carried out using boiling acetic acid (0.5 M), and the homoge-

tate was maintained at 85°C for 30 min. Such preparations yielded a predominant activity with an estimated molecular weight of M, ≈1000 (Fig. 4B). Similar results were obtained when FCS was treated with boiling acetic acid and chromatographed on Bio-Gel P-10 (data not shown). These results suggested that both bone marrow and FCS had similar heat stable activities.

**Purification and Identification of the Differentiation-inducing Factor.** The following purification procedure was carried out by homogenizing CBM (300 g) in boiling acetic acid (900 ml) as described above. The homogenate was fractionated with 2 volumes of 95% ethanol (pH 4.0), and the supernatant was evaporated; then the residue was suspended in H2O (100 ml) and heated for 10 min in a boiling water bath. The ethanol fractionation was repeated using 3 volumes of 95% ethanol. The residue was suspended in H2O (4 ml), clarified by centrifugation, and chromatographed on a Bio-Gel P-10 column (3 x 30 cm) using acetic acid (0.3 M) for elution. The fractions were evaporated, suspended in H2O, assayed for activity, and the active fractions combined (Table 2). In a preliminary experiment, we noted that the active P-10 fraction eluted from a Blue Sepharose (CL-6B) affinity column (1 x 12 cm) as a single peak at 0.4 M NaCl (0.1–0.8 M NaCl gradient in 0.02 M phosphate buffer, pH 7.5; total volume 60 ml; data not shown). However, since it was difficult to separate the salt from the preparation, we modified the elution step by washing the column consecutively with 0.1 M NaCl (in 0.01 M phosphate, pH 7.5; 20 ml), 0.3 M NaCl (in 0.01 M phosphate, pH 7.5; 20 ml), H2O (20 ml), and 0.1 M NH4HCO3 (20 ml). The NH4HCO3 fraction was lyophilized, assayed (Table 2), applied to an FPLC anion exchange column (Fig. 5), and eluted with a NaCl gradient (0–0.35 M in 0.02 M Tris, pH 7.5; 30 ml). As shown in Fig. 5, A and B, the main activity peak is associated with a corresponding 280 nm absorption peak. The purification steps and the corresponding activities are shown in Table 2. As noted in the table, substantial losses in activity occurred at each step in the purification. In part, this might have been due to our failure to recognize the hydrophobic properties of the factor and the possible consequences, particularly during such steps as drying.

We conjectured whether the low-molecular-weight UV chromophore might be TH, since it was reported that the v-erbA oncogene, a mutated avian TH receptor (5, 6) af-

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**Fig. 3.** The effect of bone marrow extract on the development of erythroid (CFU-E) colonies in methylcellulose. The experimental details are described in "Materials and Methods." The experiment was repeated three times, and a representative experiment is shown. The control with no additions showed 88 colonies and with extract (0.2 μl/ml) 52 colonies. Each well was plated with 10^3 cells/ml.

**Fig. 4.** Bio-Gel P-10 gel fractionation of the differentiation-inducing activity. Extracts were prepared from CBM by homogenizing in (A) ice-cold phosphate buffer (0.01 M, pH 7.5) or (B) "boiling" acetic acid (0.5 M) and maintained at 85°C for 30 min. After centrifugation of the homogenate, the supernatant was fractionated with three volumes of 95% ethanol (pH 4.0). The alcohol-soluble fraction was evaporated, suspended in H2O (1/10 the original volume of the homogenate), and clarified by centrifugation. This preparation (1 ml) was applied to the P-10 column (1.5 x 30 cm) and eluted with 0.3 M acetic acid. The collected fractions (1 ml) were dried and assayed for activity. Standards shown are: 1, bovine serum albumin; 2, cytochrome c; 3, vitamin B-12; 4, Cl-.

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**Table 2** Purification of the differentiation-inducing activity from bone marrow

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (μg)</th>
<th>Yield (%)</th>
</tr>
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<tbody>
<tr>
<td>Extract-1</td>
<td>2.2 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>Extract-2</td>
<td>5.0 x 10^4</td>
<td>23</td>
</tr>
<tr>
<td>Bio-gel (P-10)</td>
<td>1.15 x 10^4</td>
<td>5.3</td>
</tr>
<tr>
<td>Blue Sepharose</td>
<td>1.7 x 10^4</td>
<td>0.8</td>
</tr>
<tr>
<td>FPLC (Mono Q)</td>
<td>6.0 x 10^3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

The differentiation assay used for determining activity and units of activity are described in "Materials and Methods." Extract-1 refers to the preparation obtained after the first alcohol precipitation step in which the supernatant fraction was evaporated and the residue was suspended in H2O. Extract-2 refers to a similar preparation obtained after the second alcohol precipitation. Similar recoveries at the FPLC step had been observed with two previous preparations. Analysis of the FPLC fraction 11 indicated that it contained 45 nmol as amino acid equivalents, using the ninhydrin reaction (41) with L-leucine as a standard, and 0.15 nmol estimated as TH equivalents.
ected erythroid differentiation (7). Indeed, we found that the two active forms of TH, T₃ and T₄, were active at low physiologically relevant concentrations, as will be described in a following section. When FPLC fraction 11 was chromatographed on paper ("Materials and Methods"), the activity migrated as two separate components with Rᵢ values corresponding to T₃ and T₄ (Fig. 6). A similar chromatographic analysis of FPLC fraction 10 indicated lesser amounts (approximately 32% compared to fraction 11) of T₃ and T₄. This coinciding chromatographic behavior of the two activities and the two active iodothyronines provide strong evidence that the purified activity is TH. The presence of TH in FPLC fraction 11 was confirmed by immunoassay. Fraction 11 contained 53 ng T₄, which is a quantity in the expected range based on the clone 3–1 differentiation assay. The quantitation of the T₃ content was not reliably established due to limited amounts of sample remaining. Furthermore, the high activity shown by the synthetic TH (T₃ and T₄) in the clone 3–1 assay (see following section) reinforce the conclusion that the activity in fraction 11 is TH. Beyond the details of the identification, the implied erbA connection provides a coherent frame-

work for the understanding of the signaling pathway for erythroid development. **Biological Functions.** Recent studies have shown a close association between TH and RA activities. Both the TH and RA receptors have been shown to activate transcription through the TH response element (17). As shown in Fig. 7, clone 3–1 cells were induced by T₃ and T₄ at very low concentrations (~10⁻⁸ M), and T₃ was more active than T₄. These observations are consistent with a specific hormonal response as noted in other differentiating systems (18, 19). T₃ and T₄ also induced differentiation of parental 745A cells (Fig. 1). In addition clone 3–1 cells were maximally induced by all-trans-RA at 10⁻⁶ M (Fig. 8A). This concentration is ~100–1000 times less than the concentration previously
shown to induce the FEL cell (20) but is similar to the concentrations used in transactivation experiments (21). When RA and T₃ were applied together at maximally inducing concentrations, there was no increase in differentiation beyond that shown by the single inducer (Fig. 8B), suggesting that RA and TH may perform similar but independent functions. Both TH and RA induced terminal cell differentiation (Table 3). This is indicated by the limited cell division occurring in Bz+ colonies as contrasted to the control, noninduced colonies. Neither T₃ nor T₄ had appreciable effect on the growth of the total clone 3-1 cell population over a 72-h observation period. The cell numbers increased from 2.0 ± 0.2 × 10⁵ cells/ml at 0 time to 3.1 ± 0.9 × 10⁶ cells/ml at 72 h for cells grown in medium containing FCS. The comparable titers at 72 h for cells grown in medium containing dFCS, dFCS+T₃, and dFCS+T₄ were 2.37 ± 0.48 × 10⁵, 2.18 ± 0.44 × 10⁶, and 2.31 ± 0.76 × 10⁶ cells/ml, respectively. These results indicate that TH promotes differentiation commitment but does not stimulate proliferation.

Discussion

While our studies had been centered on the identification of the endogenous differentiation-inducing factor for the FEL clone 3-1 cell, other significant and related developments had been unfolding, particularly in the avian system. An aspect of the molecular basis for TH action was being elucidated with the discovery of the avian erythroblastosis virus oncogene v-erbA and the identification of the proto- gene product as a TH receptor and transcriptional activator belonging to the steroid hormone receptor superfamily (5, 6). It was also shown that there was functional cooperativity between the TH and RA receptors (22). We have not yet investigated the role of TH receptors in the FEL cell, but the results observed here with TH and RA are compatible with the notion of their common role in activating transcription.

What is the role of TH in the differentiation of FEL and bone marrow cells? Although FEL cells are known to be induced by a variety of agents (23–25), the extremely low concentration of TH (10⁻¹⁰ M) required to induce differentiation of clone 3-1 cells suggests a high specificity in the TH action, and its endogenous nature implies a physiological relevance. The effect of TH is not restricted to clone 3-1 because the parental cells also responded to TH, albeit at a much reduced level. TH committed clone 3-1 cells to terminal differentiation but had not marked effect on the growth of the total cell population over the period observed. In this respect, it contrasts with the effects of the colony-stimulating factors which affect both growth and differentiation of cells (26). On the other hand, the studies on bone marrow cells (27) have indicated that TH (or in our studies, the partially purified factor) did not displace the requirement for EPO but had a synergistic effect in increasing the number of erythroid colonies (Fig. 3).

A central question is whether TH functions as a physiological signal for normal erythroid differentiation. We suggest that the following considerations argue for a physiological role for TH: (a) a necessary condition for a physiologically relevant differentiation factor is its presence in the tissue or environment in which differentiation is taking effect. The identification of T₃ and T₄ as active constituents in a chromatographically purified fraction derived from a tissue known to be involved in erythropoiesis and its presence in serum at concentrations (28) which are sufficient to induce erythroid differentiation in vitro (Fig. 7) attests to this criterion; (b) the differentiation-inducing activity in serum could be substantially removed by dialysis, and the activity could be restored by the addition of TH in the media. The failure to remove all the activity by dialysis might be accounted for by the known binding of TH to various proteins in serum (29); (c) the avian system exemplified by the v-erbA oncogene not only provides the underpinning for a molecular basis for TH action but also suggests a physiological involvement of the TH receptor in erythroid differentiation (see “Introduction”). The studies reported here are complementary and suggest the commonality of the avian and murine systems; and (d) in vivo observations have indicated an association between TH deficiency and anemia in man and a correction of the anemia by TH administration (30, 31). Furthermore, in vitro studies have shown that TH stimulated erythroid differentiation (32). In these cases, TH action was considered to be indirect, and attributed to the increased production of EPO or other hemopoietic factors (33–35). However, other in
vivo studies (13, 27) including those presented here, and in vivo experiments with nephrectomized rats (36) have indicated a direct role of TH in erythroid differentiation. A direct role of TH would mean that the original observations relating TH deficiency and anemia should be reinterpreted to indicate that TH may be required for erythropoiesis. A recognition of TH as a regulatory signal for erythroid differentiation signifies the emergence of a different type of regulatory molecule in a seemingly monolithic milieu dominated by protein growth factors. TH and possibly other small, bioactive molecules may also have a niche in the differentiation pathway of hemopoietic cells.

Materials and Methods
Cells and Culture Conditions. Clone 3-1 was isolated previously as a spontaneously differentiating, anticancer drug-sensitive derivative of the parental 745A FEL cell line (15). Clone 3-1 and parental cells were cultured at 37°C in iscove's medium supplemented with 10% FCS (Bocknek; BDH, Toronto, Ontario, Canada), penicillin (50 units/ml), and streptomycin (50 μg/ml) in a humidified atmosphere containing 5% CO₂. In the differentiation assays, logarithmically growing clone 3-1 cells were centrifuged, diluted (1 × 10⁵ cells/ml), and grown in iscove's medium containing 10% Nu serum (contains 25% newborn calf serum and various additions; Collaborative Research Inc., Bedford, MA) for 3 days, at which time approximately 1% of the population is differentiated. The cells were centrifuged, washed once in serum-free iscove's medium, and suspended (1 × 10⁵ cells/ml; 0.25 ml/48-well plate) in iscove's medium containing 10% dFCS and indicated amounts of differentiation-inducing factor. After a 2-day incubation period, the cells were stained with benzidine and assayed for hemoglobin-producing, differentiated cells (at least 300 cells were counted; Ref. 37; Fig. 2). One unit of activity is defined as the amount of extract that would give one-half maximal activity. Dialyzed FCS was prepared by adjusting the pH of the serum to 4.0 with 2.0 M acetic acid, followed by dialysis against 0.02 M acetic buffer (pH 4.0) containing 0.1 M NaCl, and then phosphate-buffered saline (four changes; 48 h).

Mouse Bone Marrow CFU-E Assay. Bone marrow-derived erythroid progenitor cells were isolated from young adult CD1 mice. The cells were centrifuged and plated in methylcellulose-containing iscove's medium following the protocol described by Metcalf (16, 26). Reconstituent human EPO (0.4 units/ml; Ortho Biologics, Inc., Manati, Puerto Rico) and varying quantities of bone marrow extract-2 were included in the medium. CFU-E colonies were enumerated by direct microscopic observation after a 48-h incubation period (38) and confirmed by benzidine staining. Colonies are defined as clusters composed of eight or more cells.

The soft agar assay has been described previously (39). B2+ colonies were enumerated after 4 days in culture.

Paper Chromatography. FPLC fractions containing activity were applied as a strip to Whatman 3-mm paper and chromatographed in an ascending system using t-amyl-alcohol-n-hexane-2 M ammonia (50:10:60) as a solvent system (40). T₃ and T₄ standards (dissolved in 0.1 M NaOH or in 70% ethanol with dilute ammonia) were separately applied. The strip was developed for 32 h and dried; the standards were stained with ninhydrin. The sample-containing segments were cut into 1.5-cm strips, eluted with methanol containing 0.25% concentrated ammonia, dried in a Speed-Vac, and assayed for activity. Additional details concerning this procedure are described by Meinhold (28).

Assay for T₃ and T₄. T₃ and T₄ were assayed using a competitive immunnoassay (Chemiluminescence, Ciba Corning ACS:180 system).

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