A mutated retinoic acid receptor-α exhibiting dominant-negative activity alters the lineage development of a multipotent hematopoietic cell line

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The retinoic acid receptor (RARα) is expressed in virtually all hematopoietic lineages, but the role of this transcription factor in regulating the growth and differentiation of hematopoietic progenitors is unknown. We have constructed a mutant RARα that both exhibits dominant-negative activity against the normal RARα in transient expression assays in mouse fibroblasts and inhibits retinoic acid-induced neutrophilic differentiation of the HL-60 human promyelocytic leukemia cell line. When this dominant-negative RARα construct is introduced into the multipotent interleukin-3-dependent FDCP mix A4 murine hematopoietic cell line, there is a rapid switch from spontaneous neutrophil/monocyte differentiation to basophil/mast cell development. Thus, in this multipotent hemopoietic cell line the normal RARα transcription factor and/or related molecules appear to promote the differentiation of neutrophils and monocytes but suppress the development of basophils/mast cells.

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Retinoic acid (RA), the natural acidic derivative of vitamin A (retinol), is a critical molecule regulating growth and differentiation of a wide variety of cells. RA is centrally involved in epithelial differentiation (Lotan 1980), plays a critical role as a tissue-specific morphogen during embryogenesis (Thaller and Eichele 1987), and suppresses malignant transformation of epithelial cells both in vitro (Merriman and Bertam 1979) and in vivo (Hong et al. 1990). These diverse and complex biologic effects of RA are mediated through a number of closely related nuclear RA receptors (RARs) that are members of the steroid/thyroid hormone receptor superfamily and possess discrete DNA-binding and RA (ligand)-binding domains (Evans 1988).

The expression of RARs is widespread, with RAR mRNA noted in most fetal and adult tissue (Zelent et al. 1989). Unlike the steroid hormone receptors, the RA receptors, as well as thyroid hormone receptors, appear to be associated with chromatin and may be constitutively bound to cis-acting regulatory sequences in the absence of ligand (Lavin et al. 1988; Damm et al. 1989). Thus, these receptors may have different regulatory roles in the absence and presence of ligand.

Several lines of indirect evidence suggest that RA and RARs may be involved in regulating the development of hematopoietic progenitors. For example, RAR mRNA (predominantly RARα) is widely expressed in different hematopoietic cell types (Gallagher et al. 1989; de The et al. 1989; Largman et al. 1989). Treatment of a human myeloid leukemia cell line HL-60 with all-trans RA (ATRA) induces these cells to undergo terminal neutrophilic differentiation, and this induction is directly mediated through RARs (Breitman et al. 1980; Collins et al. 1990). In addition, human acute promyelocytic leukemia (APL) exhibits a specific 15;17 chromosome translocation involving RARα, which presumably results in the disruption of a normal, albeit unknown, regulatory function of this particular RAR (Borrow et al. 1990; de The et al. 1990, 1991; Alcalay et al. 1991; Kakizuka et al. 1991). Furthermore, ATRA induces the leukemia cells from APL patients to differentiate into mature neutrophils both in vitro and in vivo (Huang et al. 1988; Castaigne et al. 1990; Chomienne et al. 1990, Warrell et al. 1991). However, it is currently unknown whether RA and RARs might also be involved in regulating normal hematopoietic differentiation.

Dominant-negative genes encode abnormal proteins that repress the function of their normal counterparts in
a dominant manner (Herskowitz 1987). A prototype example is the v-erbA oncogene, which represses the transcription-regulating function of normal thyroid hormone receptors [encoded by c-erbA], and this contributes to the transformation of erythroblasts (Zenke et al. 1988; Damm et al. 1989). A small deletion in the carboxyl terminus of v-erbA appears to be responsible for most of the dominant-negative activity of this oncogene in transient expression assays (Damm et al. 1989; Sap et al. 1989; Zenke et al. 1990). Thus, one way to examine the role of RARα in hematopoiesis is to utilize dominant-negative receptor constructs that would suppress normal RAR function in hematopoietic precursors and then determine whether the expression of these constructs would alter the growth and differentiation of these blood cell progenitors.

In this study we have constructed a mutant RARα, which exhibits dominant-negative activity in both mouse NIH-3T3 fibroblasts and in the HL-60 human promyelocytic leukemia cell line. This construct, when introduced into the multipotent interleukin-3 [IL-3]-dependent FDCP mix A4 murine hematopoietic cell line, induces a marked switch from spontaneous neutrophil/monocyte differentiation to the development of basophils/mast cells. These observations suggest that RARs play important roles in the development of myeloid progenitors.

Results

A truncated RARα construct exhibits dominant-negative activity

As detailed in Materials and methods we created an RARα cDNA with a truncation of the sequences coding for the carboxy-terminal 59 amino acids, as well as a portion of the 3'-untranslated region. This truncated cDNA [designated RARα(403)] encodes a peptide of 403 amino acids containing the amino terminus, the DNA-binding domain, and part of the hormone-binding domain of RARα [Fig. 1A,B]. This truncated receptor was inserted into the retroviral vector LXSN [Fig. 1C] to generate the recombinant vector designated LRARα403SN [Fig. 1D]. To determine whether the truncated RARα403 construct exhibits dominant-negative activity with respect to the normal RARα, we performed trans-activation assays in mouse NIH-3T3 tk− cells. When an expression vector harboring the normal RARα gene (pEMSV–RARα) was cotransfected with a chloramphenicol acetyl transferase (CAT) reporter construct, pTRE–CAT, containing a synthetic thyroid hormone/RAR-responsive element [TRE] (Umesono et al. 1988), there was RA-induced trans-activation of the CAT reporter gene [Fig. 2A, lanes 5,6]. In contrast, when both pEMSV–RARα and the mutant LRARα403SN construct were cotransfected into the same target cells, there was a dose-dependent suppression of this RARα-mediated trans-activation [Fig. 2A, lanes 6–10]. An RARα/ RARα403 DNA ratio of 1 : 6 suppressed most of the activity of the normal RARα. When this experiment was performed using a different reporter, that is, pRRE–CAT, which harbors the natural RA-response element [RRE] of RARβ (de The et al. 1990a), there was prominent suppression of the endogenous RAR activities [Fig. 2B, lanes 3,4] and partial suppression of exogenous RARα activity. [Fig. 2B, lanes 6–9]. These results indicate that the RARα403 construct exhibits dominant-negative activity with respect to the normal RARα in mouse fibroblasts.

To determine whether this same RARα403 construct also exhibits dominant-negative activity in hematopoietic cells, we performed transient expression assays in the mouse hematopoietic cell line FDCP mix A4 [described below] by electroporation. Although the RARα403 construct suppressed some endogenous RAR activities in this cell line [data not shown], the extremely low efficiency of gene transfer achievable by electroporation in these target cells (estimated to be 1/10,000 that of NIH-3T3) made them unsuitable for adequately assessing the suppressing potential of RARα403 by transient expression. To further examine the dominant-negative function of RARα403 in hematopoietic cells, we utilized retrovirus-mediated gene transduction to introduce this mutant re-
The truncated RARα403 exhibits dominant-negative function in transient expression assays. (A) The trans-activation activities of RARα and RARα403 were studied in NIH-3T3 tk- cells in the absence and presence of 1 μM RA. An expression plasmid pTRE-CAT was used as the reporter. RARα represents the expression plasmid pEMSV-RARα; RARα403 refers to pLRARα403SN (Fig. 1D). The volume of lysate used in CAT assay was normalized for transfection efficiency determined by expression of the cotransfected growth hormone reporter pCMV-GH. The DNA ratio of pEMSV-RARα to pLRARα403SN varied from 1:9:1 to 1:9:6. pLXSN was used to equalize the total amount (2.3 μg/100-mm dish) of DNA and LTRs transfected. (+) 2.5 μg of plasmid DNA. Higher amounts (5 μg) of transfected pLRARα403SN result in suppression of >60% of endogenous RAR activity (not shown). (B) Similar to A except a different reporter [pPRES-CAT] was used in place of pTRE-CAT. (+) 1 μg of plasmid DNA. The total amount of DNA was 16 μtg/100-mm dish.

Figure 2. The truncated RARα403 exhibits dominant-negative function in transient expression assays.

Figure 3. Expression of RARα403 in HL-60 cells blunts RA-induced neutrophilic differentiation. HL-60/LXSN D.1 and D.2 are two randomly selected HL-60 clones infected with the control vector LXSN. HL-60/LRARα403SN D.2 and D.5 are two HL-60 clones infected with LRARα403SN that express high levels of the full-length 4.7-kb retroviral vector mRNA containing RARα403 sequence. Cells were induced with the indicated concentrations of RA for 5 days. Differential counts were then performed on Wright–Giemsa-stained cytospin preparations and represent the means of triplicate experiments.
These results indicate that the truncated RARα403 construct also exhibits dominant-negative activity in the HL-60 hematopoietic cell line, and its expression is not lethal in these particular neutrophil progenitors.

Characteristics of FDCP mix A4 cells

To examine the impact of the dominant-negative RARα403 on the commitment and differentiation of multipotent hematopoietic progenitors, we utilized the FDCP mix A4 cell line. This IL-3-dependent cell line was established from a murine long-term bone marrow culture and behaves like normal hematopoietic stem cells in many respects: it is nonleukemogenic, responds to several hematopoietic growth factors in a physiologically relevant manner, and spontaneously generates progenitors committed to different hematopoietic lineages [Spooner et al. 1986; Just et al. 1991]. When cultured in a medium containing horse serum and WEHI 3B cell line-conditioned medium (as a source of IL-3), the line maintained in our laboratory consists mainly of undifferentiated blasts [see Fig. 5A, below] but spontaneously differentiates into neutrophils and monocytes/macrophages (~10–30% combined) and, to a much lesser extent, into mast cells/basophils (1–2%). Northern blot analysis indicates that these cells express endogenous 3.6- and 2.6-kb RARα mRNA transcripts [Fig. 4A, lane 1]. Thus, this multipotent hematopoietic cell line appears to be well suited for investigating the impact of the dominant-negative RARα403 on hematopoietic differentiation.

Expression of RARα403 in FDCP mix A4 cells triggers the development of terminally differentiating basophils/mast cells

We infected the FDCP mix A4 cells with the amphotropic dominant-negative LRARα403SN retroviral vector, as well as the control vectors [LXSN and LRARαSN]; the latter harbors the cDNA of normal human RARα and selected the infected cells in a medium containing G418 and IL-3. Examination of Wright–Giemsa-stained cytospin preparations of FDCP mix A4 cells infected with the control retroviral vectors [LXSN and LRARαSN] revealed the predominant neutrophilic/mast cell differentiation characteristic of the uninfected parental FDCP mix A4 cells [Fig. 5A,B,D]. In contrast, a very large number of differentiated cells with numerous, intensely basophilic granules appeared in the culture infected with the LRARα403SN retroviral vector within 10 days after the infection [Fig. 5C,E]. These latter cells expressed high levels of the 4.7-kb retroviral mRNA harboring the dominant-negative RARα403 insert [Fig. 4A, lane 3]. The granules of these cells stained metachromatically with toluidine blue [Fig. 5F] and are negative for nonspecific esterase [a monocyte marker] and alkaline phosphatase [a neutrophil marker]. These morphological and cytochemical characteristics suggest that these basophilic granular cells are basophils/mast cells.

To further confirm the basophil/mast cell lineage of the basophilic granular cells in the LRARα403SN-infected FDCP mix A4 cells, we utilized flow cytometry to examine the expression of cell-surface immunoglobulin E receptor [IgE R], another basophil/mast cell-specific marker [Metzger et al. 1986; Thompson et al. 1990]. A high percentage (38.4%) of cells expressing surface IgE R was noted in LRARα403SN-infected cells, whereas only 0.4% of LXSN-infected cells were positive for IgE R [Table 1]. We also phenotyped LRARα403SN as well as the control [LXSN and LRARαSN]-infected cultures utilizing monoclonal antibodies specific for mouse neutrophils [antibody 7/4; Hirsch and Gordon 1983] and macrophages [MAC-1 antibody]. Consistent with our morphological observations, the LXSN- or LRARαSN-infected FDCP mix A4 cells contained 10–30% 7/4-positive cells, whereas the LRARα403SN-infected cultures exhibited significantly lower percentages (0–3.5%) of neutrophil antigen-positive cells [Table 1].

Histamine is a lineage-specific marker for basophils/mast cells [Riley and West 1953; Denburg 1992]. Therefore, we measured both the histamine content within these infected cells and the concentration of histamine in the culture supernatant [Table 1]. We found that the histamine content of the LRARα403SN-infected cells [2.17 pg/cell, assuming every cell contained equal amounts of histamine to allow comparison] was ~10-fold greater than the histamine content of the control LXSN-infected cells [0.25 pg/cell] and was similar to the histamine content of normal basophils/mast cells. Moreover, the histamine concentration in the supernatant of the LRARα403SN-infected cultures [3 x 10^-4 M] was ~130-fold increased over the histamine concentration in the control [LXSN]-infected cells [2.3 x 10^-6 M] [Table 1].
Figure 5. Morphology of uninfected and infected FDCP mix A4 cells. (A) Uninfected FDCP mix A4 cells. Most cells are undifferentiated blasts. The arrow points to a mature neutrophil. Arrowheads indicate differentiating cells. (B) FDCP mix A4 infected with control vector LXSN. The arrow points to a neutrophil. (C) FDCP mix A4 infected with LRARα403SN. Most cells (arrows) are differentiated and contain numerous basophilic granules and oval nuclei. (D) A higher magnification of B. (E) A higher magnification of C. (F) Toluidine blue stain of FDCP mix A4 cells infected with LRARα403SN. The granules in many cells (arrows) stain metachromatically with toluidine blue, indicating that these cells are of the basophil/mast cell lineage. (A–E) Wright–Giemsa stain. Bars, 10 μm. All except D and E have the same magnification.

We also quantitated the production of basophils/mast cells by FDCP mix A4 cells following retroviral infection and G418 selection. As shown in Figure 6, the production of basophils/mast cells in LRARα403SN-infected cultures was 20- to 70-fold higher than in LXSN-infected cultures in the first 2 weeks after the infection. These observations indicate that retrovirus-mediated gene transfer and expression of the dominant-negative RARα403 construct in FDCP mix A4 cells resulted in a switch from spontaneous neutrophil/monocyte differen-
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Table 1. Cell-surface antigen expression and histamine production by LXSN- and LRARα403SN-infected FDCP mix A4 cells

| Cell line                | Expression | Histamine |
|-------------------------|------------|-----------|
|                         | 7/4(+) | MAC-1(+) | IgE R(+) | intracellular | in supernatant |
| Experiment 1            |         |          |          |              |              |
| LXSN infected           | 28.6    | 8.3      | ND       | ND           | ND           |
| LRARα403SN infected     | 1.4     | 1.0      | ND       | ND           | ND           |
| Experiment 2            |         |          |          |              |              |
| uninfected              | 11.1    | 2.0      | ND       | 0.14         | 1.6 x 10^-6  |
| LXSN infected           | 14.5    | 2.8      | 0.4      | 0.25         | 2.3 x 10^-6  |
| LRARαSN infected        | 11.4    | 1.0      | ND       | ND           | ND           |
| LRARα403SN infected     | 3.6     | 1.0      | 38.4     | 2.17         | 3.0 x 10^-4  |

*aCell-surface antigen expression was analyzed using monoclonal antibodies specific for mouse neutrophils [7/4], monocytes/macrophages [MAC-1], or IgE receptor, as detailed in Materials and methods.

*bHistamine content was determined by radioimmunoassay. For quantification of intracellular histamine, cells were lysed in distilled water. Negative controls included NIH-3T3 fibroblast, which showed no measurable intracellular histamine, and complete culture medium, which contained <10^-9 M histamine.

Examination of Wright-stained cytospin preparations revealed very few basophils/mast cells. The LRARαSN-infected culture was indistinguishable from uninfected or LXSN-infected FDCP mix A4 cells by morphology.

Figure 6. Net production of basophils/mast cells in infected FDCP mix A4 cells. FDCP mix A4 cells [1.4 x 10^6 cells] were infected with LXSN (hatched bars) or LRARα403SN (solid bars) vectors using the supernatant infection method and selected with G418 from days 2–8. Similar percentages [10–15%] of cells infected with either retroviral vector were G418 resistant. These G418-resistant cells were subsequently maintained in culture medium supplemented with WEHI-3B-conditioned medium (as a source of IL-3) and subcultured every 3 days. Production of basophils/mast cells was calculated from the total number of cells and the differential counts on Wright-stained cytospin preparations on each feeding. The total numbers of cells (corrected for subculturing ratios) for LXSN-infected cultures were 1.3 x 10^6 (day 8), 10.1 x 10^6 (day 11), and 93.8 x 10^6 (day 14). Corresponding numbers for LRARα403SN-infected cultures were 1.3 x 10^6 (day 8), 5.5 x 10^6 (day 11), and 16.8 x 10^6 (day 14). All numbers represent mean of duplicates.

Figure 7. Growth curves of FDCP mix A4 cells infected with LRARα403SN. Cultures were initiated with 2 x 10^5 low-passage (p.7) cells of bulk cultures of FDCP mix A4 cells infected with the indicated retroviral vectors and subcultured every 3–4 days. Each data point represents the mean of duplicates.
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Observations suggest that the decreased growth rate of LRARα03SN-infected FDCP mix A4 cells might be due in part to diminished self-renewal of undifferentiated blasts. Therefore, we measured the frequency of cells capable of forming macroscopic colonies in agar cultures in the presence of IL-3. (The macroscopic colonies are defined as colonies with a diameter >0.5 mm after a 14-day incubation and are formed by cells with greater self-renewal capacity.) The frequency of macroscopic colony-forming cells of FDCP mix A4 cells infected with LRARα03SN was 0.028%, which was 18-fold lower than that of LXSN-infected cells (0.5%). Furthermore, even the macroscopic colony forming cells in the LRARα03SN-infected cultures demonstrated a relatively lower self-renewal capacity compared with those in the LXSN-infected cultures because 80% of the individual macroscopic colonies of the latter, when transferred to liquid suspension cultures, were capable of long-term continuous proliferation in IL-3-containing media while only 20% of macroscopic colonies of LRARα03SN-infected FDCP mix A4 cells demonstrated this proliferative capacity.

Examination of cytospin preparations of individual macroscopic colonies showed that all such colonies of LRARα03SN-infected FDCP mix A4 cells contained high percentages (mean 47.9%) of basophils/mast cells and lower percentages of neutrophils, monocytoid cells, and undifferentiated blasts when compared with LXSN-infected cells (Table 2). Thus, the phenotypic change we observed in the bulk culture of LRARα043SN-infected FDCP mix A4 cells was recapitulated by all clonogenic cells.

### All clonal lines of LRARα03SN-infected FDCP mix A4 cells exhibit predominant basophil/mast cell differentiation

The above analysis was performed on "bulk" cultures of G418-resistant retroviral vector-infected FDCP mix A4 cells. We also utilized soft agar cloning and limiting dilution to establish five continuously passaged clonal lines of the LRARα03SN-infected cells. All of these clones (c.1–c.5) expressed the appropriate 4.7-kb retroviral mRNA (Fig. 4A). We noted that the phenotype of these subclones was virtually identical to the bulk-infected cells. That is, all five subclones exhibited slower IL-3-dependent growth, and Wright–Giemsa-stained cytospin preparations revealed high percentages of basophils/mast cells and lower percentages of neutrophils and undifferentiated blasts in these subclones when compared with clones of LXSN-infected FDCP mix A4 cells. This phenotype is further substantiated by flow cytometry using monoclonal antibodies 7/4, MAC-1, and IgE (Table 3).

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**Table 2. Differential counts of macroscopic colonies in agar culture of LXSN- and LRARα03SN-infected FDCP mix A4 cells**

| Colony number | Basophil/mast cell (%) | Neutrophil (%) | Monocytoid (%) | Undifferentiated (%) |
|---------------|------------------------|----------------|----------------|---------------------|
| **LXSN-infected cells** |                      |                |                |                     |
| 1             | 1                      | 13             | 52             | 34                  |
| 2             | 2                      | 2              | 18             | 78                  |
| 3             | 1                      | 7              | 23             | 69                  |
| 4             | 0                      | 13             | 24             | 63                  |
| 5             | 1                      | 5              | 31             | 63                  |
| 6             | 0                      | 6              | 5              | 89                  |
| 7             | 3                      | 16             | 31             | 50                  |
| 8             | 3                      | 7              | 43             | 47                  |
| 9             | 0                      | 2              | 2              | 96                  |
| 10            | 0                      | 7              | 23             | 70                  |
| Mean          | 1.1                    | 7.8            | 25.2           | 65.9                |
| **LRARα03SN-infected cells** |                   |                |                |                     |
| 1             | 59                     | 0              | 8              | 33                  |
| 2             | 51                     | 0              | 8              | 41                  |
| 3             | 26                     | 0              | 7              | 67                  |
| 4             | 52                     | 3              | 17             | 28                  |
| 5             | 50                     | 3              | 15             | 32                  |
| 6             | 59                     | 2              | 17             | 22                  |
| 7             | 25                     | 3              | 30             | 42                  |
| 8             | 66                     | 0              | 11             | 23                  |
| 9             | 60                     | 2              | 14             | 24                  |
| 10            | 31                     | 0              | 37             | 32                  |
| Mean          | 47.9                   | 13             | 16.4           | 34.4                |

Colony assays were performed as described in Materials and methods. Individual macroscopic colonies (>0.5 mm diam.) were identified with a dissecting microscope, and cytospin preparations were made of individual colonies and stained with Wright–Giemsa stain. Differential counts were performed on 100 cells each.

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FDCP mix A4 cell line is by no means the equivalent of the lineage development of these cells. Although the hematopoietic cell line results in a dramatic change in many of these cells.

In this study we have observed that introducing a mu- other basophil/mast cell characteristics displayed by the IL-3-dependent, multipotent FDCP mix A4 murine mutant RARαexhibiting dominant-negative activity into 

### Discussion

Enhanced GATA-1 expression in LRARα403SN-infected FDCP mix A4 cells

GATA-1 is a zinc finger-containing transcription factor that is preferentially expressed in hematopoietic cells of the erythroid, megakaryocyte, and mast cell lineages but not in neutrophilic or monocytic lineages (Tsai et al. 1989; Martin et al. 1990). Although GATA-1 was initially identified as a transcriptional regulator of globin gene expression, recent studies show that GATA-1 also binds to the promoter region of the gene of mast cell-specific carboxypeptidase A and activates its expression (Zon et al. 1991). We utilized Northern blot hybridization to compare GATA-1 mRNA levels in the LRARα403SN-infected FDCP mix A4 cells versus the control (LXSN)-infected cells. Relatively low levels of GATA-1 mRNA were detected in the LXSN-infected FDCP mix A4 cells. In contrast, a marked increase in steady-state GATA-1 mRNA was noted in the LRARα403SN-infected bulk cultures as well as the five individual infected subclones (Fig. 4B). This enhanced GATA-1 mRNA expression noted in the LRARα403SN-infected FDCP mix A4 cells further corroborates the other basophil/mast cell characteristics displayed by many of these cells.

| Clonal line          | [IgE R(+) (%) | 7/4(+) (%) | MAC-1(+%) |
|----------------------|--------------|------------|-----------|
| **LXSN-infected cells** |              |            |           |
| c.1                  | 0.0          | 17.4       | 7.4       |
| c.2                  | 6.2          | 23.1       | 9.1       |
| c.3                  | 3.9          | 21.2       | 29.0      |
| c.4                  | 4.0          | 24.2       | 7.9       |
| c.5                  | 3.6          | 17.8       | 8.5       |
| Mean                 | 3.5          | 20.7       | 12.4      |
| **LRARα403SN-infected cells** |            |            |           |
| c.1                  | 47.4         | 1.9        | 0.9       |
| c.2                  | 54.4         | 1.3        | 0.9       |
| c.3                  | 66.4         | 0          | 0         |
| c.4                  | 56.3         | 0          | 0         |
| c.5                  | 74.9         | 0          | 0         |
| Mean                 | 59.9         | 0.6        | 0.4       |

Clonal lines were established by limiting dilution subcloning in 96-well plates or in soft agar. Flow cytometry was performed as described in Materials and methods.

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Table 3. Cell-surface antigen expression by clonal lines of FDCP mix A4 cells infected with LXSN and LRARα403SN retroviral vectors

Enhanced GATA-1 expression in LRARα403SN-infected FDCP mix A4 cells continues to produce a small number of neutrophils and monocytes in addition to the large number of basophils/mast cells (Tables 2 and 3) indicates that the individual clonogenic cells are at least tripotent [i.e., capable of differentiating into (1) basophils/mast cells, (2) neutrophils, and (3) monocytes]. Therefore, the observed phenotypic change does not result from immortalization of any rare committed, unipotent basophil/mast cell progenitors. [In fact, the basophils/mast cells in LRARα403SN-infected cultures have a limited life span of 6–12 days and degenerate thereafter.] Rather, this phenotype is the result of a shift by the multipotent FDCP mix A4 cells from the spontaneous production of primarily neutrophils and monocytes to the continuous, preferential production of basophil/mast cells. It should be emphasized that the net production (absolute number) of basophils/mast cells produced by LRARα403SN-infected FDCP mix A4 cells is increased by 20- to 70-fold within the first 2 weeks of infection. [Fig. 6]. This finding makes it unlikely that the altered FDCP mix A4 phenotype is the result of selective elimination of neutrophils and monocytes in the culture resulting in the progressive accumulation of basophils/mast cells. In this latter scenario, the percentage of ba-
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basophils/mast cells would increase in the infected cultures, but the absolute number would not.

Concomitant with the switch from spontaneous neutrophil/monocyte differentiation to the development of basophils/mast cells, the LRARα403SN-infected cells exhibit lower percentages of morphologically undifferentiated blasts (Table 2), display an 18-fold reduction in the frequency of macroscopic colony-forming cells, and show a markedly reduced growth rate (Fig. 7). These three parameters suggest that the self-renewal of LRARα403SN-infected cells has decreased, either owing to the prolongation of the cell cycle of undifferentiated blast cells, to the increased recruitment of these cells into the terminal differentiation pathway, or both. The effects of the dominant-negative LRARα403 on the development of different myeloid lineages and the self-renewal of FDCP mix A4 cells are schematically summarized in Figure 8.

Our observation that introducing a dominant-negative RARα construct into the FDCP mix A4 cells results in a dramatic change from spontaneous neutrophil and monocyte differentiation to basophil/mast cell develop-

ment indicates that the normal RARα plays important roles in regulating the development of myeloid progenitors. It appears that normal RARα promotes the development of neutrophils and monocytes but represses the development of basophils/mast cells. One possible model is that the normal RARα blocks the development of basophils/mast cells by uninfected FDCP mix A4 cells. Removal of this blockade by the dominant-negative LRARα403 allows the development along the basophil/mast cell lineage under the strong influence of IL-3 used to maintain the culture. (IL-3 is a potent inducer of basophil/mast cell differentiation by normal hematopoietic stem cells.) In this model, the normal RARα does not necessarily play the role of a “master switch” that commits cells to different specific hematopoietic lineages. More likely, this transcription factor plays some important role or roles in the complex cascade of molecular events leading to or subsequent to the lineage commitment process. It is important to note that this apparent hematopoietic regulatory activity of RARα occurs in the relatively low concentrations of RA that are endogenous to serum (<10^{-8} M; DeRuyter et al. 1979; Cullum and Zile 1986). (There is 20% horse serum in our complete culture media.) This suggests that RARα exerts its hematopoietic regulatory activity in FDCP mix A4 cells either in the absence of or in relatively low concentrations of ligand. In contrast, significantly higher concentrations of RA [10^{-7} to 10^{-5} M] are required to induce the differentiation of promyelocytic leukemia cells (Breitman et al. 1980; Chomienne et al. 1990) or to activate genes at the 5’ end of the Hox-2 cluster in human embryonal carcinoma cells (Simeone et al. 1990).

Although it is clear from cotransfection studies in NIH-3T3 fibroblasts [Fig. 2] that the RARα403 construct exhibits dominant-negative activity against the normal RARα, the full spectrum of its biological activity is yet to be defined. The prototype dominant-negative nuclear receptor is the v-erbA oncogene, which exhibits deletions in the carboxy-terminal ligand-binding domain compared with its homolog c-erbA, the thyroid hormone receptor. These carboxy-terminal deletions abolish T₃ binding to the v-erbA protein, and as a result v-erbA protein may interfere with normal thyroid hormone function by forming heterodimers with c-erbA that cannot be activated by T₃ (Damm et al. 1989; Desbois et al. 1991). Our RARα403 construct involves a 59-amino-acid truncation in the carboxy-terminal RA binding domain, which most likely also interferes with ligand [RA] binding. Thus, RARα403 may interfere with normal RARα function by forming inactive heterodimers with RARα. Alternatively, the mutated RARα may compete for interactions with other transcription factors that may be essential for RARα activity. For example, members of the RXR family of RA receptors can dimerize with RARs, and these heterodimers display greater affinity for specific DNA target sequences than do RAR homodimers (Kliwer et al. 1992; Leid et al. 1992; Yu et al. 1992; Zhang et al. 1992). The RARα403 gene product potentially may form inactive heterodimers with RXRs, and this sequestration of RXRs may prevent the latter

\[ A \] Normal RARα

\[ B \] RARα Suppressed

Figure 8. Schematic summary of the effects of the dominant-negative LRARα403 on the lineage development of FDCP mix A4 cells. [A] When RARα is presumably functioning normally, the IL-3-dependent FDCP mix A4 cells exhibit prominent self-renewal and a predominance of spontaneous differentiation along the neutrophil and monocyte lineages (solid arrows). Spontaneous differentiation to basophil/mast cell lineage is suppressed [broken arrows]. [B] In FDCP mix A4 cells infected with the dominant-negative LRARα403SN retroviral vector, the normal RARα function is presumably suppressed. These cells now exhibit prominent spontaneous differentiation to basophils/mast cells, and differentiation to neutrophils and monocytes is suppressed. The infected cells remain IL-3 dependent, but self renewal is markedly diminished.

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from potentiating the activity of other RA receptors. Of relevance is the finding that a truncated construct containing the first 404 amino acids of RARs (differing from our RARa403 construct by only a single amino acid) can dimerize with RXRs (Yu et al. 1992). Thus, it is possible that the dominant-negative RARa403 exerts its biologic effects not only by directly inhibiting the normal RARa but also by indirectly inhibiting related receptors whose full function depends on dimerization with RXRs.

The data reported here indicate that in the multipotent IL-3-dependent FDCP mix A4 murine hematopoietic cell line, RARa and/or related molecules positively influence the development of neutrophils and monocytic cells, as well as the self-renewal pathway, but repress the development of basophils/mast cells. We do not know whether RARa directly participates in this hematopoietic lineage commitment process or, alternatively, is involved in regulating the differentiation after lineage commitment has occurred. In either case, the alteration in the lineage development of the multipotent FDCP mix A4 hematopoietic cell line following the transduction of the dominant-negative RARa construct indicates that RARa and/or closely related molecules play important roles in the development of neutrophils, monococytes, and basophils/mast cells.

Materials and methods

Plasmid constructions

The sequence of full-length cDNA of human RARa has been published (Giguere et al. 1987). The plasmid pGEM3Z–hRARa DS′ harbors human RARa cDNA sequences from the initiator ATG to the BamHI site at position 2103. This plasmid was digested with Smal, which cut at position 1311 and 1597 of RARa cDNA (Fig. 1A). The larger fragment was isolated, ligated with an Nhel linker (which inserted a stop codon), and recircularized. The new construct was designated pGEM3Z–RARa403 [Fig. 1B]. An EcoRI site was present in the pGEM3Z polylinker immediately 5′ to the initiator ATG of the RARa403 insert. The 1.8-kb EcoRI–BamHI fragment of pGEM3Z–RARa403 was then cloned into the EcoRI–BamHI site of the retroviral vector LXSN [Fig. 1C] (Miller and Rosman 1989), and the recombinant vector was designated pLRARa403SN [Fig. 1D]. The plasmid pLRARaSN was constructed by subcloning a 2.8-kb EcoRI fragment of human RARa cDNA clone [Giguere et al. 1987] into the EcoRI site of pLXSN as detailed previously [Collins et al. 1990]. The expression plasmid pEMSV–RARa was constructed by cloning the 2.8-kb human RARa cDNA into the EcoRI site of the expression vector pEMSVscrib [Davis et al. 1987]. Plasmids pTRE–CAT and pRRE–CAT consist of a single copy of either a synthetic palindromic TRE [5′-TCAGGTCATGACCTGA-3′] [Umesono et al. 1988] or the RRE identified in the RAR8 promoter [5′-GTAGGGTTCACCGAAAGTTCACTC-3′] [de The et al. 1990a] inserted upstream of the thymidine kinase promoter [−109 to +5] and CAT reporter gene and was obtained from Jeannette Bigler [Fred Hutchinson Cancer Research Center]. Plasmid pCMV–GH was constructed by inserting a 1.1-kb PstI–XbaI fragment containing the cytomegalovirus immediate early gene promoter from pON249 [Geballe et al. 1986] into the human growth hormone reporter construct p6GH (Nichols Institute, San Juan Capistrano, CA) and was provided by Ed Agura (Fred Hutchinson Cancer Research Center).

Transient expression assays

NIH-3T3 tk− cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and plated 24 hr before transfection. The medium was replaced with fresh DMEM supplemented with 10% charcoal–resin-stripped FCS 4 hr before transfection. Cells were transfected by calcium phosphate precipitation with 2.5 μg of pEMSV–RARa, varied amounts of pLRARa403SN, 2.5 μg of pTRE–CAT, 1 μg of pCMV–GH as an internal control for transfection efficiency, and pLXSN to equalize the total amount [23.5 μg/100-mm dish] of DNA and long terminal repeats (LTRs) transfected. For transient expression assays using pRRE–CAT as the reporter, the following conditions were employed: 1 μg of pEMSV–RARa, varied amounts of pLRARa403SN, 4 μg of pRRE–CAT, 1 μg of pCMV–GH, and pLXSN to equalize the total amount [16 μg/100-mm dish] of DNA and LTRs transfected. Sixteen hours after transfection, the cells were washed with PBS and refed with DMEM supplemented with 10% charcoal–resin-stripped FCS and incubated for another 32 hr with or without 10−6 M ATRA. Electroporation of FDCP mix A4 cells was performed in 0.4-mm cuvettes using a Bio-Rad gene pulser [Richmond, CA] with the following parameters: 2 × 107 cells, 25 μF/800 V, 75 μg each of pCMV–GH, pRRE–CAT (containing four copies of RRE instead of one), pEMSV–RARa and pLRARa403SN. Culture supernatants were collected for determination of growth hormone concentration using a radioimmunoassay kit [Nichols Institute]. Preparation of cell lysates and CAT assay were performed according to published methods [Sambrook et al. 1989]. The volume of cell lysate used in the CAT assay was normalized for transfection efficiency as determined by the growth hormone reporter (pCMV–GH) internal control. Following thin layer chromatography on silica gel, the nonacetylated and acetylated 14C-labeled chloramphenicol was quantitated by a PhosphorImager and ImageQuant software [Molecular Dynamics].

Retroviral producer cell lines and retroviral infection

Amphotropic viral producer cell lines were established according to published procedures [Miller and Rosman 1989]. Briefly, pLRARa403SN plasmid was transfected into the ectopic viral packaging cell line PE501, and the transiently expressed viral particles were rescued to infect the amphotropic viral packaging cell line PA317 [Miller 1990]. Infected PA317 cells were selected in G418-containing medium, followed by subcloning. One clone, PA317/LRARa403SN c.10, expressing high levels of retroviral mRNA of the predicted size by Northern blot analysis and producing supernatants with a titer of 4 × 106 to 6 × 106 colony-forming units (CFU)/ml, as assayed on NIH-3T3 tk− cells was chosen as the producer of retroviral vector particles for this study. A cell line, PA317/LRARaSN producing a retroviral vector [LRARaSN] harboring the cDNA of the normal human RARa was established similarly as described previously [Collins et al. 1990].

HL-60 and FDCP mix A4 cells were infected with retroviral vectors by either a 24-hr cocultivation with irradiated [1200 rads] viral producers or infection with viral supernatants in the presence of 4 μg/ml of polybrene. Infected cells were then selected in complete culture medium containing 1 mg/ml [for HL-60] or 300 μg/ml [for FDCP mix A4] of G418 for 8–10 days. Clonal lines were generated by limiting dilution in 96-well plates or cloning in 0.3% agar.

Cell cultures

HL-60 cells were maintained in RPMI 1640 supplemented with 5% FCS. FDCP mix A4 cells were maintained in Iscove's mod-
ified Dulbecco’s medium (IMDM) supplemented with 20% (vol/vol) horse serum and 10% (vol/vol) WEHI 3B cell line-conditioned medium (as a source of IL-3) and subcultured every 3 days. Colony assays of A4/LXSN and A4/LRARα403SN were done in IMDM supplemented with 25% horse serum, 10% WEHI 3B-conditioned medium, and 0.3% agar. These agar cultures were incubated at 37°C in 5% O2, 5% CO2, and 90% N2 for 14 days.

**Northern blots**

Total RNAs were extracted with guanidine hydrochloride and subjected to Northern blotting in formaldehyde gels as described previously (Collins et al. 1987). Molecular probes used in Northern hybridization included the following: hRARα, the 1.3-kb EcoRI–SmaI fragment of the phk1 RARα cDNA clone (Giguere et al. 1987), and the mouse GATA-1 cDNA probe (Tsai et al. 1989), which was obtained from David Martin (Fred Hutchinson Cancer Research Center). All probes were labeled with 32P by nick translation before hybridization.

**Cell phenotyping**

Cytospin preparations of cell suspensions were stained with 1% toluidine blue for 15 min and washed with tap water. For quantitation of intracellular histamine, 2 × 10⁶ cells were washed with PBS and resuspended in 1 ml of distilled water and lysed by three cycles of freezing and thawing. Radioimmunoassay of histamine in cell lysates or culture supernatants was performed according to the manufacturer’s instructions (Immunotech International).

For flow cytometry, cells were exposed to saturating concentrations of primary rat IgG2a monoclonal antibodies 7/4 (generously provided by S. Gordon, Oxford, UK) or MAC-1 (Pharmigen) at 4°C for 10 min, washed with PBS/1% FCS, and stained with an FITC-labeled goat anti-rat IgG (H + L) for 10 min at 4°C. After final washing with PBS/1% FCS, fluorescence was measured with a Becton–Dickinson FACScan flow cytometer and analyzed by the Reproman software (Kirkegaard & Perry Labs, MD). An irrelevant rat IgG2a monoclonal antibody (Pharmigen) was used as the isotype control. For IgE R staining, cells were incubated with culture medium containing 15 µg/ml of mouse IgE (Pharmigen) at 37°C overnight, washed three times with PBS/1% FCS, stained with FITC-goat anti-mouse IgE (Nordic Immunologicals) at 4°C for 10 min, followed by washing with PBS/1% FCS.

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